UNIVERSIDADE DE LISBOA

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SHORT-TERM BEHAVIOURAL REACTIONS OF TWO DOLPHIN SPECIES TO A BIOPSY POLE SYSTEM: A PRELIMINARY ASSESSMENT OF ANIMAL WELFARE AND TECHNIQUE VALIDATION

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DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

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LISBOA

Ao meu pai

Aknowledgments

À minha mãe, essa fonte inesgotável de carinho e de apoio, que sempre me deu asas para poder fazer os meus “passeios” pelo mundo mesmo que isso significasse voar para longe dela. Sempre serás o meu porto de abrigo, obrigada por tudo.

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Short-term behavioural reactions of two dolphin species to a biopsy pole system: a preliminary assessment of animal welfare and technique validation

Abstract

Free-ranging cetaceans are fully adapted to aquatic environments, and thus can act as sentinels of the ecosystem's health and guide conservation policies. The Chilean dolphin (*Cephalorhyncus eutropia*) and the Peale's dolphin (*Lagenorhynchus australis*) are two coastal species of small cetaceans whose distribution greatly overlaps with human activities. Understanding the species ecology, biology, physiology and health status is vital to properly direct conservation efforts. Tissue samples can provide a lot of insight and that has been achieved through the development and improvement of biopsy techniques. This preliminary study aims to determine the short-term effect of a biopsy pole sampling protocol on the welfare of these two species of small cetaceans through a behavioural assessment.

The skin samples were taken within the Chiloé Marine Ecoregion, Chile, between December and April of 2015, 2016 and 2017. Combining observational efforts and video records, we were able to successfully register 32 individual reactions (IR) and 26 group reactions (GR) from the Chilean dolphin, and 75 individual reactions and 63 group reactions from the Peale's dolphin. Their behavioural response was classified according to a continuous numerical scale (0= no noticeable reaction, 1= startle, 2=mild, 3=moderate, 4= strenuous reactions).

Significant statistical differences ($p<0.05$) were found between species regarding the individual and the group reaction. For the Chilean dolphin the most commonly seen individual reaction was level 2 (90.6%) followed by level 1 (9.4%). The group reactions presented high correlation with the individual reactions, displaying level 2 responses in 80.8% of the biopsy attempts and level 1 reactions in the remaining 19.2%. The Peale's dolphin presented more heterogenous responses. Concerning the individual reaction, level 2 was the most frequently seen (64.0%), followed by level 1 (28.0%) and finally level 0 (8.0%). The group reaction consisted mainly in level 2 (41.3%), followed by 0 and 1 (both at 28.6%) and lastly level 4 (1.5%).

Most biopsies induced only mild individual and group reactions, which translated into a low short-term impact on the behaviour of these two dolphin species. By only producing a light disturbance on biologically essential activities for their survival and reproduction, these results support that this skin biopsy method can be used as a safe and minimally invasive technique, suitable to maintain the welfare of the individuals sampled and their populations.

Key words: cetaceans, Chilean dolphin, Peale's dolphin, biopsy sampling, behaviour, welfare

Reações a curto-prazo, de duas espécies de golfinhos, a uma técnica de biópsia com lança adaptada: análise preliminar de bem-estar e validação da técnica

Resumo

Os cetáceos de vida livre encontram-se perfeitamente adaptados ao ambiente aquático, podendo assim actuar como sentinelas da saúde do ecossistema e guiar políticas de conservação. O golfinho chileno (*Cephalorhyncus eutropia)* e o golfinho-do-sul *(Lagenorhynchus australis*) são duas espécies costeiras de pequenos cetáceos cujas distribuições se sobrepõem a actividades humanas. Entender a ecologia, biologia, fisiologia e o estado de saúde destas espécies é vital para que se consigam, adequadamente, direcionar os esforços de conservação. A obtenção de amostras de tecidos pode gerar bastante conhecimento e tem sido possível através do desenvolvimento e aprimoramento de técnicas de biópsia. Este estudo preliminar visa determinar o efeito, a curto prazo, de um protocolo de biópsia com recurso a uma lança adaptada, sobre o bem-estar destas duas espécies através de uma avaliação comportamental. As amostras cutâneas foram recolhidas na Ecorregião Marinha Chiloense, entre dezembro e abril de 2015, 2016 e 2017. Combinando esforços observacionais e registos em vídeo, reunimos com sucesso 32 reacções individuais (RI) e 26 reacções do grupo (RG) do golfinho chileno, e 75 RI e 63 RG do golfinho-do-sul. A reacção comportamental dos animais foi classificada de acordo com uma escala numérica contínua (0 = sem reação perceptível, 1 = sobressalto, 2 = reacção leve, 3 = reacção moderada, 4 = reacção vigorosa).

Foram encontradas diferenças estatísticas significativas ($p < 0{,}05$) entre ambas as espécies quanto à reacção individual e a grupal. Para o golfinho chileno, a RI mais frequentemente observada foi de nível 2 (90,6%), seguida pelo nível 1 (9,4%). As RG assemelharam-se bastante às IR apresentando respostas de nível 2 em 80,8% dos eventos e reacções de nível 1 nos restantes 19,2%. O golfinho-do-sul apresentou um leque mais heterogéneo de respostas. Em relação às RI, o nível 2 foi o mais frequente (64,0%), seguido pelo nível 1 (28,0%) e, finalmente, pelo nível 0 (8,0%). A RG exibida foi maioritariamente de nível 2 (41,3%), seguido pelos níveis 0 e 1 (ambos a 28,6%) e, por último, pelo nível 4 (1,5%).

A maioria das biópsias induziu apenas reações leves quer a nível individual quer grupal, o que se traduziu num reduzido impacto a curto-prazo no comportamento de ambas as espécies de golfinhos. Dado que o distúrbio produzido sobre as actividades, biologicamente, importantes para a sobrevivência e reprodução das espécies foi considerado mínimo, os nossos resultados defendem que esta é uma técnica de biópsia segura e minimamente invasiva, apropriada à manutenção do bem-estar dos indivíduos amostrados e das suas populações.

Palavras-chave: cetáceos, golfinho chileno, golfinho-do-sul, biópsia, comportamento, bem-estar

Table of Contents

List of Figures

List of Tables

List of Graphics

List of Abbreviations and Symbols

% - Percentage

cDNA – Complementary deoxyribonucleic acid

DD – Data Deficient

DNA – Deoxyribonucleic acid

e.g. – exempli gratia

GR – Group Reaction

h – Hour(s)

IR – Individual Reaction

IUCN - International Union for Conservation of Nature

m – Meter(s)

mg – Milligram(s)

min – Minute(s)

mm – Millimetre(s)

NT – Near Threatened

WHO - World Health Organization

RNA – Ribonucleic acid

spp. – species

ppm – parts per million

UNAB – Andrés Bello University

U.S. – United States

Internship short report

This study was performed during the 6th year of the Integrated Masters in Veterinary Medicine of the Faculty of Veterinary Medicine, Lisbon University, under the supervision of Dr. Cayetano Espinosa-Miranda, scientific director of NGO YaquPacha-Chile and co-supervised by Professor Dr. Virgílio Almeida (Faculty of Veterinary Medicine, Lisbon University).

The curricular internship took place between January 10th and May 1st 2016, performing a total of 840 hours. The training period took approximately 2 months in the Chilean Patagonia, with most of the surveys taking place in the southeast of Chiloé Island and being completed with two expeditions to the continental fjords around Chaitén and Melimoyu areas. During this period I lived on site, in a wooden house by the bay where our zodiac was anchored.

The *Chiloé Small Cetaceans Project* focuses on the conservation ecology of dolphins and porpoises and the study of anthropogenic threats to the marine ecosystem in the Chiloé Archipelago in northern Patagonia, Chile. Their flagship species (Figure 1) are the Chilean dolphin (*Cephalorhynchus eutropia*), the Peale's dolphin (*Lagenorhynchus australis*), and the Burmeister's porpoise (*Phocoena spinipinnis*). Through research and education, they aim to promote conservation of ecologically and economically important coastal ecosystems.

Figure 1 - The project's three flagspecies. Top left the Chilean dolphin; top right the Peale's dolphin; and bottom left the Burmeister's Porpoise. On the bottom right an example of an outreach activity performed at an indigenous school. Photo credits: YaquPacha ©.

The work consisted mainly in learning and perfecting tools used for wild small-cetaceans conservation. The supervisor was undertaking his PhD under the title "The Effect of Anthropogenic Coastal Stressors on the Skin Microbiome and Immune Expression of Small Cetaceans: Elucidating threats from an ecoimmunological perspective" and thus arose the need to perform skin biopsies from these dolphins. Given that both species of dolphins are poorly known and had never before been biopsied with this method, we decided to record the behavioural reactions of these animals to the sampling procedure in order to evaluate our impact on their welfare.

During the summer field season, I learned how to manoeuvre the 4 meter dinghy, how to collect environmental data (e.g. salinity, depth, water visibility, temperature) and animal data (e.g. photo I.D.; size and constitution of the dolphin group). Collecting data was just the beginning of long evenings organizing it, transferring it to different databases and finally analysing it. In various occasions I had to plan, prepare and perform environmental education classes for the indigenous communities in the surrounding areas. Sharing the importance of ocean conservation in general and teaching about the species they share their homes with in particular, were common themes in our classes. However, the one that gave me the most joy was the building of a paper microscope and subsequent collection of different types of marine tissues to be observed under the microscope (Foldscope ©)

Figure 2 - Environmental education class using Foldscopes © (paper microscopes). On the top left image there is me on my first attempt to assemble the microscope. On the bottom left there is a kid visualizing its algae sample on the foldscope. On the right image you can see a young girl half way through the assembly of her own paper microscope.

The last month was spent at the Ecology and Natural Resources Faculty laboratory, from Andrés-Bello University in Santiago, Chile. The tissue samples collected in Patagonia were first processed here: organization and classification of the samples, DNA and RNA extraction and isolation, cDNA synthesis and cDNA quantification.

To add to my training, I had the opportunity to help in a different field of conservation medicine, taking action in the rescue and rehabilitation of endemic Chilean fauna (but not restricted to) at UFAS, the Wild Fauna Rehabilitation Unit from Andrés Bello University and Buin Zoo. There I worked under the supervision of Dr. Nicole Sallaberry-Pincheira, teacher at the university and director of the rehabilitation centre.

I worked with species such as: South American grey fox (*Lycalopex griseus*), Andean fox (*Lycalopex culpaeus*), Austral Pygmy-Owl *(Glaucidium nana*), White-tailed kite (*Elanus leucurus leucurus)*, Variable hawk (*Geranoaetus polyosoma*), Chimago (*Milvago chimango*), Aplomado falcon *(Falco femoralis*), Barn owl (*Tyto alba*), Magellanic Horned owl (*Bubo magellanicus*), Lesser Grison (*Galactis cuja*), Elegant Fat-tailed Mouse Opossum (*Thylamys elegans*), amongst others (Figure 3).

During a double two-week period, I was directly or indirectly involved in many activities such as:

- The daily visit and maintenance of the animals' different enclosures;
- Food preparation and stocking;
- Patients' daily physical exams; weekly blood draws and biochemical exams;
- Whenever required coprology exams were undertaken as well as samples collected for bacteriology/virology/cytology/histopathology/parasitology;
- Wound care and bandaging. Drug administration (intravenous, intramuscular);
- Administration of fluids for patient stabilization (subcutaneous and intravenous).
- Assessment and treatment of ocular pathologies in raptors;
- Anesthesia of raptors (Figure 4);
- Assistance in the collection of radiographs from raptors and foxes and ultrasounds from the latter;
- Assistance in osteopathic surgeries, particularly in raptors with broken wings after being shot or electrocuted;
- Microchipping all animals before their release;
- Bird's beak and nail trimming, whenever needed;
- Necropsy of a dozen species of birds, reptiles and small mammals.

Furthermore, I had the opportunity to participate in the "Avian Medicine & Surgery Workshop: Wild and Exotic Birds". The theoretical component consisted of two days (12th and 13th January) in the University of Andrés Bello campus (UNAB) and the practical component consisted of two days (14th and 16th January) split between the UNAB Zoological Medicine Hospital and the Rescue Centre UFAS. There I attended classes of Avian Anatomy and Physiology; Raptor Medicine; Avian Wound Healing; Avian Emergencies; Avian Radiology; Infectious Diseases; Parasitology; Geriatrics; Zoonotic Diseases and Ethics of Wildlife Rehabilitation.

The practical component of the workshop was divided in four chapters. In the first one I got to learn practical techniques for avian handling and physical examination, as well as learn practical techniques for laboratory sample collection, basic treatment procedures, bandaging, grooming, and placement of tube & Elizabethan collars. The second section was dedicated to anaesthesia procedures and radiology positioning. In the third chapter I learned about soft tissue and orthopaedic surgery, and finally the last one was about avian pathology laboratory and necropsy techniques.

Figure 3 - Several Argentinian tortoises (*Chelonoidis chilensis*) were being collected from all over Chile to reintroduce in Argentina.

Figure 4 - Inducing anaesthesia on a black-chested buzzard-eagle (*Geranoaetus melanoleucus*) that was electrocuted and needed soft tissue surgery to its right wing.

General introduction

Marine mammals are fully adapted to aquatic environments. Given that they depend on the health of these ecosystems for their survival, the study of these species will truthfully reflect the ecosystem's vulnerabilities and level of deterioration (Moore, 2008). Acting as sentinels of the ocean's status, cetaceans can guide human stewardship activities towards conservation and subsequently towards promoting human health (Bossart, 2011).

The Chilean dolphin (*Cephalorhyncus eutropia*) and the Peale's dolphin (*Lagenorhynchus australis*) are two species of small cetaceans native of south waters of South America (Viddi & Harcourt, 2016), and whose distribution greatly overlaps with human activities (e.g. fisheries, mussel farms, fish farms and transportation vessels).

It is known that human activities can directly or indirectly, intentionally or unintentionally, have a negative impact on cetaceans' well-being (de Vere, Lilley & Frick, 2018). Assuring good animal welfare should always be a priority, given that the health of the ecosystems are intimately dependent upon the health of the species it comprises (Aguirre, O'Hara, Spraker & Jessup, 2002).

Ocean pollution, both chemical and physical, such as plastic debris or ghost fishing gear, is currently one of the hottest topics discussed worldwide. Cetaceans, as top predators, bioaccumulate chemical pollutants which can cause endocrine disruption, reproduction impairment and immune disfunction (Fossi et al, 2004). Plastic debris cause harm mainly by ingestion, which consequently may lead to a disfunction in digestion and absorption culminating in starvation, or by entanglement, which can cause severe tissue damage if not death (de Vere et al., 2018). Fishery interactions can pose a serious risk to marine mammals either through bycatch and vessel strikes, which are usually lethal, or by disturbance of behaviour due to high traffic intensity and habitat overlap, which may impair the animal's welfare (Sai Leung & Leun, 2003). Anthropogenic noise, due to vessel traffic, sonar activity, drilling and seismic surveys, just to name a few, can be heavily deleterious on the acoustic communication between cetaceans, which depend upon it for a myriad of activities (social interactions, navigation, echolocation, foraging, and reproduction). As exemplified, the health of marine mammals is being disrupted by human activities, thus raising to the importance of studying these animals in order to preserve its populations viability and promote conservation strategies.

Studying behaviour and welfare is important to research marine mammals. Cetaceans are highly cognitive and socially complex. Understanding that behavioural funnelling can lead to vulnerability, while behaviour flexibility may help a species resilience, leads us to acknowledge the importance of incorporating behavioural data into conservation management (Brakes & Dall, 2016). Poor welfare might induce an impairment in reproduction, growth rate, and immunity, which consequently may lead to increase morbidity/mortality, ultimately affecting the populations fitness (Dawkins, 2003). Measuring welfare both at an individual and population levels is crucial because effects on individual animals can be assessed immediately while impacts on populations can only be measured after a considerable period of time, when irreversible changes might potentially have already taken place (Papastavrou, Leaper & Lavigne, 2017).

Assuring the well-being of a species under research is of maximal importance. Especially when referring to endangered and small sized populations, in which the individual value of each animal increases exponentially and as such its individual welfare too. The best current illustration is the critical scenario faced by the Vaquita porpoise (*Phocoena sinus).* This species is the most endangered marine mammal in the world as a result of bycatch in gillnets. Despite efforts by the Mexican government in banning fishing activities over the distribution of the vaquita, continued decline forced a more intrusive approach. The conservation plan aimed for the capture and maintenance under human care of the last 30 individuals, keeping them in a temporary sanctuary until it would become safe to be released back into the Gulf of California. In the meantime, a breeding protocol would be put in place to increase population size. However, even with all the experts and state-of-the-art equipment, the rescue efforts had to be halted due to the death of a female vaquita after capture. It appears that these animals react poorly to being in a new environment (Vaquita CPR, 2017). Critical cases such as this demonstrate the importance and need for the understanding of a species behaviour and welfare requirements.

With the aim of studying the effects of human stressors in the two Patagonian dolphin species immune systems, the need for collecting skin biopsy samples arose. Overall considered to be a minimally invasive technique, biopsy sampling can still pose risks (Noren & Mocklin, 2012). In 2000, Bearzi reported the death of a common dolphin (*Delphinus delphi*) as a consequence of possible vertebral trauma or stress. This example further confirms that behaviour is not only species specific as it may also reveal individual variation. Since this was the first time this biopsy sampling technique was applied on both species, Chilean and Peale's dolphins, there was a clear concern for the animals' well-being. Attending to the possible detrimental effects on the animals' welfare, we decided to make a behavioural assessment on the animals' response to the procedure. Although it is impossible to protect wild animals from all kinds of negative welfare issues, as veterinarians we have the moral responsibility to prevent unnecessary harm whenever possible and reduce the inevitable ones.

CHAPTER I – Literature Review

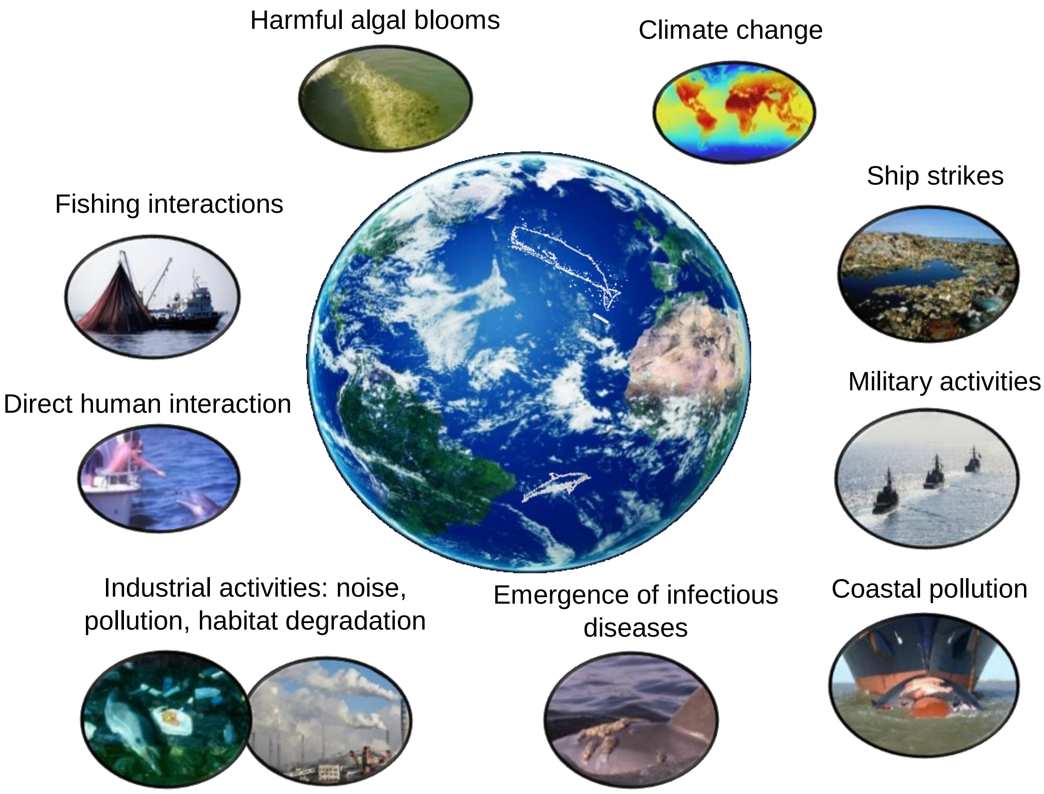
1 - Cetaceans as sentinel species for marine ecosystem health

According to the Convention on the Conservation of Migratory Species of Wild Animals (CMS, 2014), cetaceans face an array of threats, from direct catch and by-catch, vessel traffic and noise disturbance to habitat degradation, over-fishing, pollution and climate change (Figure 5). From all these, the major threat for odontocetes has been by-catch in fishing operations, as stated by the Marine Mammal Commission in 2014: "bycatch and entanglement in fishing gear remain the greatest source of direct marine mammal mortality in the United States and around the world".

Reeves, McClellan and Werner (2013) brought to attention how little and fragmented is the information available regarding the real bycatch number, urging to address this issue with the right methodologies towards the protection and restoration of marine mammal diversity and subsequent ecological health. Therefore, in recent years there have been increasing efforts to investigate the impact of fisheries on cetaceans and how to mitigate it (Brown, Reid & Rogan, 2013, 2015; Stolen et al., 2013; Marçalo et al., 2015; Di Tullio, Fruet & Secchi, 2015).

We now admit the risks dolphins currently face are all directly or indirectly caused by anthropogenic activities (Culik, 2004; Van Bressem et al., 2009; Jepson & Law, 2016). Therefore, understanding these threats and developing a holistic management based on scientific evidence, becomes vital to protect and preserve these species, as well as the marine environment in which they inhabit (Bossart, 2011).

Figure 5 - List of threats affecting cetacean's health and survival.



The World Health Organization (WHO) in its constitution defines health as a "state of complete physical, mental and social well-being and not merely the absence of disease and infirmity" (WHO, 1948). Extrapolating this to the level of an entire ecosystem, the idea of "health" becomes an integrative concept in which complex systems maintain intimate interrelationships between organisms (humans included), as well as with the abiotic factors of the environment they are in (Aguirre et al., 2002).

Rapport (1989) was the first to propose the concept of ecosystem health, and later Costanza and Mageau (1999) summarized the attributes comprising his concept as follows: (1) homeostasis; (2) absence of disease, (3) vigour or scope for growth and reproduction; (4) stability or resilience (capacity to buffer perturbations); (5) diversity or complexity; and (6) balance between system components.

The oceans cover approximately 70% of the Earth's surface, providing approximately 60% of the biosphere's economic value and representing direct subsistence for over 200 million people (Wilcox & Aguirre, 2004). The marine environment has constantly been under human pressure, especially during these last couple of decades, leading to its alteration and degradation. We now admit the ocean's health is indivisibly connected to human and animal health. This triangle of Environment, Animal and Human common health issues has been referred to as ''one health, one medicine'' (Bossart, 2011; van Helden, van Helden, & Hoal, 2013). In 2005, the 32nd report from the U.S. Marine Mammal Commission stated that threats to marine mammals are ultimately related to the size, growth rate, consumption patterns and behaviours of humans. This assumption reminds us that advocating for marine mammal health is in our best interest, given how it can affect us (Stewart et al., 2008).

In order to assess the quality of health in the marine environment, scientists started surveying sentinel species (Wells et al., 2004; Aguirre & Tabor, 2004). The word "sentinel" derives from the Italian word "sentinella" that derived itself from the Latin verb "sentire" which means "to perceive/to feel". Sentinel is thus defined as "someone that has to stand guard and keep watch over something". Therefore, a sentinel species would detect changes to the environment before the effects become noticeable to us or worst, irreversible (Reddy, Dierauf & Gullard, 2001). The National Research Council (1991) defined an animal sentinel system as a "system in which data on animals exposed to contaminants in the environment are regularly and systematically collected and analysed to identify potential health hazards to other animals or humans". These early warnings enable primary responses to potentially harmful conditions and allow an effective management of resources (Bonde, Aguirre & Powell, 2004).

Holden (1972) was probably the first scientist to use marine mammals as environment sentinels. In his study he monitored organochlorine contamination of the marine environment through the analysis of residues in seals. Marine mammals are highly adapted and specialized to the environment they inhabit and from which their survival depends. They are crucial in the maintenance of the structure and function of pelagic marine ecosystems (Méndez-Fernandéz, Polesi, Taniguchi, Santos & Montone, 2016). As such, free-living cetaceans, as marine mammals, are a logical choice as sentinels of the ocean's health. It is important to note that captive or stranded animals are not representative of natural populations (Wobeser, 1994).

Several studies (Katona & Whitehead, 1988; Bowen, 1997; Aguilar, Borrell & Pastor 1999; Reddy et al. 2001; Aguirre & Tabor, 2004; Burek, Gullard, & O'Hara, 2008; Moore, 2008; Smee, 2010; Bossart, 2011; Brito & Sousa, 2011) assembled scientific evidence that justify the use of free-ranging cetaceans as sentinels:

1) They are "charismatic megafauna" that as a special public appeal and can be more effective at drawing social attention and action to the plight of ecosystems (so called flagship species).
2) They depend on the marine environment to survive, which is linked to its integrity. As such, changes in the environment can severely impact their populations.
3) Some species are migratory which allows us to have access to information on a wide geographical area.
4) They have long life spans and are at the highest trophic level, thus they are more likely to show the biomagnification effects of contaminants.
5) They can reflect the status of inferior trophic levels, given they are top predators.
6) They have large blubber stores that can serve as depots for anthropogenic chemicals and toxins.
7) Many marine mammal species share the same coastal environment as humans, consuming the same food and thus being important to assess potential public health threats.

According to Moore (2008), selecting the appropriate marine mammal species to use as a sentinel of change depends on the ecological alteration of concern. Migratory mysticete whales may be used to investigate broad scale shifts in ecosystems, whereas coastal dolphins are suited for the monitoring of pollutants, disease vectors, or anthropogenic activities in nearshore habitats.

2 - Biopsy sampling as a research tool

In marine mammals, the most commonly used and easiest method for obtaining biological samples is to resort to naturally stranded or incidentally captured animals (Méndez-Fernandez, Polesi, Taniguchi, Santos & Montone, 2016). Although it has the advantage of retrieving large amounts of tissues, in general the animals who strand are either ill or their carcass is in an emaciated and/or deteriorated condition. Additionally, these individuals are not randomly selected and as such are not representative of natural populations (Wobeser, 1994). Due to this unreliability, researchers have developed non-lethal sampling techniques to study free--living marine animals (Noren & Mockling, 2012).

Unlike terrestrial animals, obtaining blood samples from cetaceans is logistically challenging and restricted to unique situations. Therefore, the skin is the easiest and most accessible tissue to acquire, making it the preferred type of sample. Important to note that when obtaining a skin sample, it is also easy to collect a hypodermis sample, augmenting our opportunities to generate understanding.

The skin is a critical interface between mammals and its external environment, which prevents the loss of moisture, regulates body temperature, receives environmental stimuli, reabsorbs lipophilic substances, protects deeper tissues, blocks the entry of pathogens, and synthesizes pre-vitamin D_3, that is important for bone formation as well as the function of brain, heart, muscles and immune system (Mostafa & Hegazy, 2015). Genetic analyses of skin samples can provide information on population parameters as individual gender and identification, social organisation, population size, movement patterns, mating system and stock identity, genetic philopatry and variability (Amos & Hoelzel, 1990; Waples, 1991; Baker et al., 1990, 1993; Bérubé et al., 1998; Palsbøll, Bérubé, & Jørgensen, 1999; Möller & Beheregaray, 2004; Sellas, Wells & Rosel, 2005; Knoff, Hohn & Macko, 2008; Caballero et al., 2012; Louis et al., 2014). Furthermore, the population's sex ratio can be estimated through molecular sex determination (Quérouil et al., 2010; Kellar et al., 2013). Examining the blubber portion of the samples can lead us to determine pollutant concentrations (Marsili & Focardi, 1996; Fossi et al., 2000, 2004; Berrow et al., 2002; Fair et al., 2010; Sinclair et al., 2015; Balmer et al., 2018), and pregnancy status (Trego, Kellar & Danil, 2013; Clark et al., 2016). It also allows to learn the animal's feeding ecology and its nutritive condition through the examination of stable isotopes, fatty acids and lipid content (Herman et al., 2005; Valenzuela, Sironi, Rowntree & Seger, 2009; Gross, Kiszka, van Canneyt, Richard & Ridoux, 2009; Kiszka, Oremus, Richard, Poole & Ridoux, 2010; Wilson, Nelson, Balmer, Nowacek & Chanton, 2013).

In sum, the skin is a vital organ to the immune, endocrine and nervous systems. Therefore, the knowledge generated through skin biopsy samples constitutes an important first step in elucidating the relationship between the animal health, animal-environmental interactions, and potentially, ecosystem health (Bierlich et al., 2018).

Remote biopsy sampling is a widely recognized technique for obtaining skin and blubber tissues from free-ranging cetaceans (Noren & Mocklin, 2012) and has successfully been used on both mysticetes and odontocetes (Aguilar & Nadal, 1984; Mathews, Keller & Weiner, 1988; Weinrich, Lambertsen, Baker, Schilling & Belt, 1991; Barrett-Lennard, Smith & Ellis, 1996; Gauthier & Sears, 1999; Crain et al., 2014; Fruet et al., 2017).

Samples have been obtained using crossbows or compound bows (Lambertsen, 1987; Mathews et al., 1988; Whitehead, Gordon, Matthews & Richard, 1990; Palsbøll, Larsen & Hansen, 1991; Weinrich et al., 1991, 1992; Hooker, Baird, Al-Omari, Gowens & Whitehead, 2001; Jefferson & Hung, 2008; Kiszka, Simon-Bouhet, Charlier, Pusineri & Ridoux, 2010), rifles or pneumatic guns (Lambertsen, Baker, Weinrich & Modi, 1994; Krützen et al., 2002; Parsons, Durban & Claridge, 2003), pole systems (Fossi et al., 2004; Bilgmann, Griffiths, Allen & Möller, 2007), skin swabs (Milinkovitch, Dunn & Powell, 1994; Harlin, Würsig, Baker, & Markowitz, 1999), and by picking up sloughed skin (Amos & Hoezel, 1990; Amos et al., 1992; Valsecchi, Glocknbr-Ferrari, Ferrari & Amos, 1998) or doing faecal collection (Parsons et al., 1999, 2003; Green, Herzing, & Baldwin, 2007). Each method presents its own potentials and limitations. The two latter ones are considered non-invasive methods because they do not cause any physical lesion, and as such do not affect the animal's health, which translates into minimal ethical implications. Furthermore, both are quite simple and low cost techniques. Its disadvantages lie in the difficulty of obtaining sufficient and quality material and easiness in it getting contaminated. Skin swabbing does not allow for most toxicological and feeding analysis tests, and faecal sampling is quite time consuming (waiting for the animal to defecate) and also complicated because dolphins defecate underwater which rapidly gets diffused, requiring close approximation of the snorkelers to the group (Harlin et al., 1999; Green et al., 2007). On the other hand, there are the invasive methods, which by collecting skin and blubber samples allow for most toxicological and feeding analysis tests, besides the genetic analysis, but do cause tissue damage, which may pose a risk for infection. Crossbows and riffles require more expensive material and are considered remote methods, which means they do not require a close approximation to the group, however the sampling is of higher impact when compared to the pole system (Weller, Cockcroft, Würsig, Lynn, & Fertl, 1997). The latter although low cost and easy to perform, requires the animals to bow-ride, which limits the species it can be performed on (Loizaga de Castro, Hoelzel & Crespo, 2013). There is still little information regarding infection risks and behavioural impact on the mid and long-term on these still considered minimally invasive methods.

Attending to the fact that these procedures involve a degree of intrusion or disturbance, efforts have been made to minimise the impact on the animal's well-being (Weinrich et al., 1991; Clapham & Mattila, 1993; Brown, Corkeron, Hale, Schultz & Bryden, 1994; Patenaude & White, 1995; Gauthier & Sears, 1999).

3 - Animal welfare

When collecting tissue samples from live animals, concerns about their welfare arise. If not from an ethical and moral point of view, because the quality of the results will depend upon the quality of the animals (Castle, 2016).

Awareness to the importance of animal welfare considerations emerged after the publication of Ruth Harrison's book called ''Animal Machines'' (1964), which discussed how often animal production industry mistreated animals. The year after, the British government assembled the Brambell Committee where the concept of the "Five Freedoms" was born. These outlined five aspects of animal welfare under human control and has since been adopted by the Farm Animal Welfare Committee (FAWC, 2009):

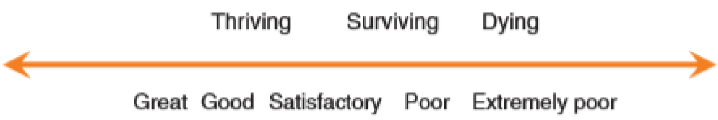
* Freedom from hunger and thirst
* Freedom from discomfort
* Freedom from pain, injury and disease
* Freedom to express normal behaviour
* Freedom from fear and distress

Although this was an important step towards the beginning of the animal welfare debate, we cannot assess the quality of welfare of a particular individual based only upon granting its five freedoms, because these are not precise enough, and do not allow for a scientific measurement (Broom, 2011).

The Freedoms concept should only be used as an initial indication of what should be assessed and what should be provided to the animals. Broom and Johnson (1993) defended that determining the animal's needs is far more important and can transform welfare into a measurable scientific concept. For them, "needs" are requirements, fundamental in the biology of the animal, to obtain a specific resource or answer to a particular environmental or bodily stimulus. As such, needs are not all of the same importance. Food and water are fundamental needs whereas a comfortable lying area may be considered less important (if considering animals under human care).

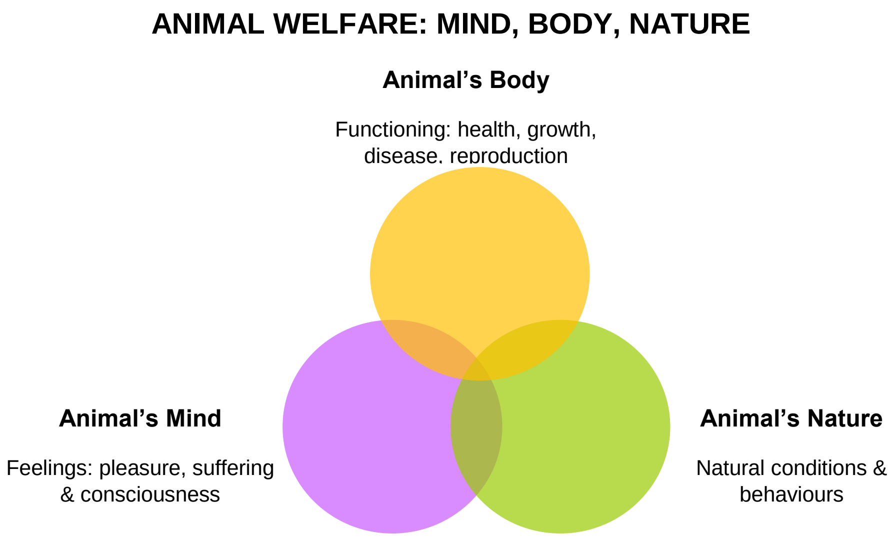
In 1986 Broom suggested "welfare of an individual [to be] its state as regards its attempts to cope with its environment". Subsequently, coping could be translated to "having control of mental and body stability" (Broom & Johnson, 1993). With this definition, welfare could now be scientifically measured, ranging between "very poor" and "very good" (Figure 6). Nonetheless, it can only be assessed in relation to one individual at a time, given that it can vary between animals of the same species, regardless of being exposed to the same environment (Hill & Broom, 2009). It should also be determined at a species level given the members of such species evolved together to adjust to particular situations (e.g. environment, dietary needs, etc.).

Figure 6 - Animal welfare can be viewed and judged along a continuum, with the extremes representing great and extremely poor welfare. These states are respectively associated with animals that are thriving in their environment or may die because they are no longer able to cope (Melfi, 2009).



While there are experts like McGlone, who in 1993 suggested that an “animal is in poor state of welfare only when physiological systems are disturbed to the point that survival or reproduction are impaired”, there are other welfare scientists who prefer to emphasise the psychological aspects of animal welfare, which likely echo motivational and physiological states (Veasey, Waran & Young, 1996). In 1990, Dawkins stated that any individual who’s experiencing an unpleasant mental state, will have its welfare compromised. Later, in 2006, she acknowledged that although the feelings of an animal should be the centre, its health would also be an important welfare indicator. Duncan (1993) shared the same idea by defending that “neither health nor lack of stress nor fitness is necessary and /or sufficient to conclude that an animal has good welfare. Welfare is dependent upon what the animals feel”. For Fraser (2008) animal welfare had to be considered as a broad concept which could be grossly grouped under three main captions: the basic health and functioning of animals (“body”), their affective states (“mind”) and the possibility for animals to live reasonably natural lives (“nature”), all together constituting the Three Orientations Model (Figure 7). This notion was adopted in 2010 by the American College of Animal Welfare (ACAW) which determines that in order to assess welfare we need to consider the “animal’s health, behaviour, and biological function” (ACAW, 2018).

Figure 7 - Animal welfare embraces three holistic components, the animal’s body, mind and nature (adapted from Fraser, 2008, and Melfi, 2009).



Recently, and aiming towards that same holistic perspective on welfare, Mellor (2016a) decided to update the Five Freedoms paradigm to the Five Provisions aligned with the Animal Welfare Aims. With this upgrade he intended to minimise negative experiences or states (as the previous model already envisioned) and actively promote positive ones (highlighting the animal's affects). To this end, the Animal Welfare Aims were introduced to redesign the Five Freedoms, as the Five Provisions constitute the requirements to achieve such welfare goals (Mellor, 2016b; Table 1).

Table 1 - The updated Five Provisions and aligned Animal Welfare Aims from Mellor (2016b).

Provisions	**Animal Welfare Aims**
1. Good nutrition: Provide ready access to fresh water and a diet to maintain full health and vigour	Minimise thirst and hunger and enable eating to be a pleasurable experience
2. Good environment: Provide shade/shelter or suitable housing, good air quality and comfortable resting areas	Minimise discomfort and exposure and promote thermal, physical and other comforts
3. Good health: Prevent or rapidly diagnose and treat disease and injury, and foster good muscle tone, posture and cardiorespiratory function	Minimise breathlessness, nausea, pain and other aversive experiences and promote the pleasures of robustness, vigour, strength and well co--ordinated physical activity
4. Appropriate behaviour: Provide sufficient space, proper facilities, congenial company and appropriately varied conditions	Minimise threats and unpleasant restrictions on behaviour and promote engagement in rewarding activities
5. Positive mental experiences: Provide safe, congenial and species-appropriate opportunities to have pleasurable experiences	Promote various forms of comfort, pleasure, interest, confidence and a sense of control

Most welfare studies have been focused on identifying signs indicating poor welfare, admitting that good welfare would result from an absence of suffering (Clegg & Delfour, 2018). However, it is now accepted that the measurement and promotion of good welfare and positive emotions should be as important as the prevention of negative ones (Mellor, 2016a).

The welfare needs of free-living animals are insufficiently defined and, as such, the welfare of wild animals ultimately depends upon stakeholders' personal relationships, of the way they value and relate with animals (Miller, Anthony & Golab, 2018).

As presented, Animal Welfare is a rather new subject in Science and thus its idea is still not unanimously defined. Nevertheless, it will always be a concept in constant evolution, adjusting to changing cultures, to Science improvements and to the emergence of new methods to measure physiological, behavioural and cognitive parameters.

4 - Measuring behaviour as a welfare assessment

Over the past few years an increased concern about non-domestic animals' welfare has been noticed (Miller et al., 2018), however there is still a huge gap when referring to wild species. Very little is known about the biology and ecology of many groups and what is known is generalized across species (Melfi, 2009). This proved to be true in our study. The Peale's dolphin is still considered Data Deficient on IUCN's redlist and the Chilean dolphin, although already categorised as Near Threatened, it is still poorly known. Furthermore, when working with threatened species, higher caution needs to be taken given any loss or long term detrimental effects on welfare produced by our research could have a huge impact on the survival of such species.

According to Melfi (2009) and Hill and Broom (2009) the most commonly used measures of welfare can be divided into three sections:

1- Health measures, such as growth, health status, reproduction rate and longevity;
2- Physiological measures, such as heart and respiratory rates, and the hypothalamic--pituitary-adrenal axis activity, which is typically monitored through cortisol or corticosterone levels.
3- Behavioural measures, such as changes in normal behaviours; presence/development of stereotyped behaviours; self-directed displacement; change in activity budgets; assessment of behavioural diversity and comparison with the wild.

Behaviour assessments can be good indicators of animal welfare since they may give us a good perception on if the animal is able to adjust well to its environment, through the choices it makes and reactions it has to a variety of stimuli. Nonetheless it is important to emphasize how many animals have evolved to hide any indication of poor welfare, given it would be disadvantageous to communicate to any potential predators or competitors that such animal is experiencing any hardships (Hill & Broom, 2009). This is true for dolphins, where pain is difficult to assess given they mask their symptoms for adaptive reasons (Clegg & Delfour, 2018). Also, several indicators of poor welfare may only be revealed at a late stage, which means we may have compromised welfare for quite some time until we are able to notice it.

To evaluate behaviour, it is essential to know the normal range of behavioural activity of the species under observation, as well as the spectrum of individual variability. Attempting to define what's normal *versus* abnormal can be quite a challenge, open to interpretation (Miller et al., 2018). Behavioural assessments are in part a subjective activity, which makes harder to prove its validity.

Most research has been conducted on terrestrial animals, specially domesticated species, given their economic interest and availability. Furthermore, the majority of welfare assessments has been conducted on terrestrial mammals, lacking standard guidelines for free-ranging marine animals, hence the importance of studies like ours.

According to Dawkins (2006) good animal welfare begins by assuring physical health but it also implies that animals have positive emotions over negative ones. In the wild, animals experience negative emotions, like hiding or escaping predators, on which they do not possess control over, but there are also cases where animals deliberately choose to sacrifice their own welfare in order to assure the perpetuity of its species (Dawkins, 1998). This is the case, for example, of when penguins starve themselves to nurse their young while their partner is out at sea feeding itself.

In sum, even though researchers try to ensure the best welfare by performing wild-captive comparisons, there are several conceptual and procedural issues to it (Veasey et al., 1996; Miller et al., 2018):

- Observers may influence behaviour of wild more than of captive animals;
- There are a number of abiotic and biotic factors that might influence animal behaviour (e.g. health status, age, weather);
- Behaviours seen may not be representative, since sample sizes of both wild and captive animals may be small because of rarity and difficulty in access;
- Variability between different populations, both in the wild and in captivity, may distort results;
- Same behavioural measures are seldom used (observers are never the same in the wild as in captivity);
- Assumption that welfare in the wild is optimal.

Even though they are easier to perform, and less invasive, behavioural assessments are difficult to interpret and may be considered less rigorous, as such, several scientists would rather complete the welfare assessment based on physiological measures. However, in the wild, being able to collect the samples required for such analysis (blood, faeces, urine, etc.) might prove to be quite hard and even produce biased results.

Animal welfare and conservation are interconnected, with actions to improve welfare having the possibility to promote conservation outcomes (Gales et al., 2009). In 2007, Broom made a very pertinent statement that if one would want to assess the quality of life of a certain individual, one would need to assess its welfare over a lengthy period of time. Once again reflecting the importance of long-term studies and the lack of such in wild animals.

It is possible to mitigate our impact on cetacean's welfare through rigorous surveillance with systematic scientific data collection and analyses on a variety of welfare indicators from both short and long-term ranges (de Vere et al., 2018). These are essential to evaluate and improve the performance of human activities against target species. Furthermore, and given that our research could have some negative impact on the dolphins' welfare, we pushed for the training and education of all personnel involved in any sort of human-dolphin interaction.

5 - Somatosensory perception in cetaceans

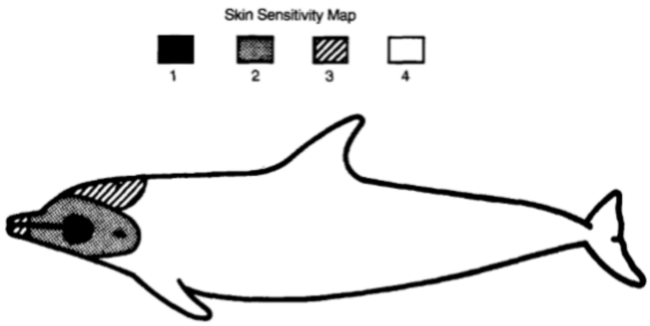
The survival of any organism depends upon their sensory perception of what surrounds them. From being able to find food, communicate and recognise their affiliates, detect the potential presence of a predator or identify any significant changes in their physical environment, it all is of the utmost importance for a species endurance.

Dolphins evolved from a terrestrial to an aquatic environment about 47 - 50 million years ago apparently from a small tetrapod ancestor (Thewissen, Cooper, George & Bajpai, 2009). With such a radical change in environment, it obviously triggered extensive changes in the animals' anatomy, physiology and behaviour (Gatesy et al., 2013). The ocean environment is a highly mutable one. There are constant changes in pressure, tides, currents and even temperature, to which the animals inhabiting it must be able to perceive in order to adjust accordingly. To this end vertebrates have developed several different receptor types (mechanoreceptors, nociceptors, and thermoreceptors) located in their dermis, muscle and joints. All together they form a somatosensory system, providing the animal the ability to feel temperature (thermoreception), touch (mechanoreceptors), pain (nociception), and perceive body position (proprioception).

Given cetaceans evolved to be more streamlined and hydrodynamic, they have lost their sensory hairs or vibrissae. Most odontocetes only possess vibrissae on the new-born's rostrum, losing them shortly after birth (Kremes et al., 2016). Therefore, the dolphins' skin is a well inervated organ and very sensitive to touch (Tyack, 2000).

Lende and Welkar (1972) were the first to study dolphin skin sensitivity, recording Somatosensory Evoked Potentials, followed by Kolchin and Bel'kovich a year later (1973), who studied the Galvanic Skin Response. Both tried to develop a map of body skin sensitivity (Figure 8). They found that dolphins are most sensitive around the blowhole and eyes, followed by snout, lower jaw and melon, reaching a sensitivity comparable to human fingertips or lips (Ridgway & Carder, 1990). In these areas they can perceive pressures as small as 10mg/mm^2 (Kolchin & Bel'kovich, 1973).

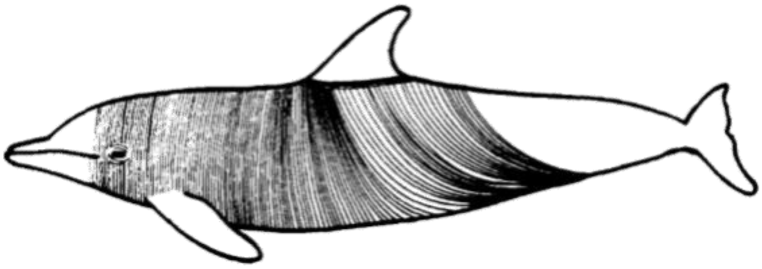
Figure 8 - Map of skin sensitivity based on Somatosensory Evoked Potentials. The belly and genital areas were not tested. 1, most sensitive, followed by 2, 3 and 4 in descending order (Ridgway and Carder, 1993).



The least sensitive part of their body seems to be along the back, like around the posterior area to the dorsal fin (Ridgway & Carder, 1993).

The skin of bottlenose dolphins is covered by small ridges (Figure 9) that are approximately circumferentially oriented in the anterior part of the body, from head to dorsal fin, and somewhat more obliquely positioned in the posterior part of the body, from dorsal fin to caudal fin (Kremers et al., 2016). These ridges have been suggested to be involved in the sense of touch (Shoemaker & Ridgway, 1991), hydrodynamics (Ridgway & Carder, 1993) or both.

Figure 9 - Orientation of cutaneous ridges on a bottlenose dolphin (Ridgway & Carder, 1993).



Touch is important for short-range communication between animals. It is used in a variety of contexts, from play and sexual displays to maternal care and social encounters (both in aggressive and affiliative situations). Rubbing one another is an important social part of dolphin's behaviour. Contact swimming, gentle stroking with the pectoral fin or rubbing against another individual. It all seems to maintain affiliative relationships in some dolphin species, the equivalent of social grooming in primates (Tyack, 2000).

6 - Behavioural reactions to biopsy sampling

As biopsy sampling has become more widely used, there is also growing concern regarding the detrimental effects it may have on the sampled cetaceans. Reactions to the biopsy procedure have been studied on 7 species of baleen whales (mysticeti) and 2 species of toothed whales (odontoceti), showing little/minimal behavioural effect or disturbance (Table 2)**.**

Table 2 - Summary of a variety of biopsy methods used and behavioural responses for whale species (concised and adapted from Noren & Mocklin, 2012).

Whale species	Biopsy method	Behavioural response	Reference
Blue whale (*Balaenoptera musculus*)	Dart delivered by draw crossbow, without retrieval line	31.1% responded and most showed low reaction. No strong reactions.	Gauthier & Sears (1999)
Fin whale (*Balaenoptera physalus*)	Dart delivered by crossbow, without retrieval line	Responses none to low level (startle).	Jahoda et al. (1996) Gauthier & Sears (1999) Fossi et al. (2003)
Gray whale (*Eschrichtius robustus*)	Dart delivered by crossbow, without retrieval line	Minimal reaction and short lived.	Mathews (1986)
Humpback whale (*Megaptera novaeangliae*)	Dart delivered by draw crossbow, with retrieval line	In general low to moderate reactions. Some strong reactions when the dart wouldn't get loose.	Weinrich et al. (1991, 1992) Lambertsen et al. (1994)
	Dart delivered by draw crossbow, without retrieval line		Weinrich et al. (1991) Clapham & Matilla (1993) Brown et al. (1994) Gauthier & Sears (1999) Cantor et al. (2010)
Minke whale (*Balaenoptera acutorostrata*)	Dart delivered by draw crossbow, without retrieval line	84% responded. Most ranged from low to moderate reactions.	Gauthier & Sears (1999)
North Atlantic right whale (*Eubalaena glacialis*)	Dart delivered by crossbow or compound bow, with retrieval line	Only 20.5% of the animals responded and most showed a minimal reaction and short lived.	Brown et al. (1991)
Northern Bottlenose whales (*Hyperoodon ampullatus*)	Dart delivered by crossbow	89% responded but most with minimal reaction and all were short lived.	Hooker et al. (2001)
Pigmy blue whale (*B. musculus brevicauda*)	Dart delivered by pneumatic gun, with retrieval line.	None to low level responses.	Kato et al. (1996)
Southern Right whales (*Eubalaena australis*)	Dart delivered by riffle or pneumatic gun.	All responses were either none or low. There were no strong reactions.	Kato et al. (1996) Best et al. (2005)
Sperm whale (*Physeter macrocephalus*)	Dart delivered by draw crossbow.	All reacted in a short-term startle reaction, with increase in speed.	Whitehead et al. (1990)

For these large species, there seems to be inconsequential, short-term behavioural disturbances (Jefferson & Hung, 2008). Mainly startle reactions, with low-to-moderate amplitude, although the level of reaction varies slightly between species, and also between populations and individuals (Kowarski, Augusto, Frasier & Whitehead, 2014). Evidences even suggest that skin sampling does not cause any long-term adverse effects, such as individual or herd displacement from a specific geographic region (Weinrich et al., 1991; Clapham & Mattila, 1993; Bown et al., 1994; Gauthier & Sears, 1999; Hooker et al., 2001; Best et al., 2005; Cantor, Cachuba, Fernandes & Engel, 2010).

Observation of reactions by small cetaceans (delphinids) are similar, reporting short-term and mild behavioural reactions (Aguilar & Nadal, 1984; Barrett-Lennard et al., 1996; Weller et al., 1997; Krützen et al., 2002; Parsons et al., 2003; Bilgmann et al., 2007; Gorgone, Haase, Griffith & Hohn, 2008; Jefferson & Hung, 2008; Kiszka, Simon-Bouhet, et al., 2010; Tezanos-Pinto & Baker, 2012; Loizaga de Castro et al., 2013), without evidence of long-term impact (Table 3).

Table 3 - Summary of some biopsy methods used and behavioural responses for dolphin species (modified from Noren & Mocklin, 2012).

Dolphin species	Biopsy method	Behavioural response	Reference
Bottlenose dolphin (*Tursiops truncatus*)	Dart delivered by crossbow	Hits: produced moderate reactions and limited in duration. Misses: 40% produced low level reactions and the rest, 60%, had no reaction.	Weller et al. (1997)
	Dart delivered by riffle	Mainly mild to moderate short-term reactions, irrespective to being hit or missed.	Krützen et al. (2002)
	Dart delivered by crossbow	Mainly low to moderate responses. One strong reaction.	Berrow et al. (2002)
	Dart delivered by pneumatic projector	Responses ranged from mild to moderate.	Parson et al. (2003)
	Pole with biopsy tip	Main reaction was mild followed by no reaction. There were no strong reactions.	Bilgmann et al. (2007)
	Dart delivered by crossbow	Mainly mild reactions.	Gorgone et al. (2008)
	Dart delivered by riffle	Mainly mild reactions (to both hits and misses, and both individual and group responses)	Tezanos-Pinto & Baker (2012)
Dusky dolphin (*Lagenorhynchus obscurus*)	Pole with biopsy tip	Individual reactions were generally mild and short-term or none.	Loizaga de Castro et al. (2013)
Guiana Dolphin (*Sotalia guianensis*)	Dart delivered by crossbow	No changes in group structure, habitat use patterns or behaviour towards the boat.	Cunha et al. (2010)
Indo-Pacific Humpback Dolphin (*Sousa chinensis*)	Dart delivered by crossbow	Mostly slight reactions with a few moderate ones (startle reaction to both hits and misses); all short-term. No strong reactions.	Jefferson & Hung (2008)
Killer whale (*Orcinus orca*)	Dart delivered by pneumatic dart projector, without retrieval line.	Most reacted only with momentary shakes or acceleration at the surface, but did not changed activities, group formation or travel direction.	Barrett-Lennard et al. (1996)
Short-beaked common dolphin (*Delphinus delphis*)	Pole with biopsy tip	Main reaction was stage 2 (mild) out of 4 followed by no reaction (level 0). There were no strong reactions.	Bilgmann et al. (2007)
		Little or no reaction	Aguilar (pers. comm, IWC 1991)
	Spear gun	Moderate reaction	
	Crossbow	Strong reaction	
Striped dolphin (*Stenella coeruleoalba*)	Pole with biopsy tip	All responses either none to low level (e.g. temporary vessel avoidance).	Aguilar (pers.comm, IWC 1991) Hohn (pers.comm, IWC 1991) Marsili & Focardi, (1996) Fossi et al. (2004)
	Dart delivered by pneumatic riffle	No significant alterations in swimming patterns.	Aguilar & Nadal (1984)

In sum, the results from seven species of small odontocetes are consistent with large whales, mainly producing mild behavioural effects in the form of short-term reactions from the biopsied animal.

For small cetaceans, there are ample data on remote biopsy sampling systems (Weller et al., 1997; Krützen et al., 2002; Gorgone et al., 2008; Jefferson & Hung, 2008; Kiszka, Simon-Bouhet et al., 2010; Quérouil et al., 2010; Tezanos-Pinto & Baker, 2012), but there are only a few published studies that examined the behavioural response to a biopsy pole system (Marsili & Focardi, 1996; Fossi et al., 2004; Bilgmann et al., 2007; Loizaga de Castro et al., 2013). In these four experiments the results suggest the biopsy pole technique generally elicits mild/moderate behavioural responses, whether the individuals are hit or missed, and that the different species (striped, bottlenose, dusky and short-beaked common dolphins) react similarly to the sampling procedure. They generally sped up and swam away from the vessel, but it was possible to approach several sampled individuals closely again within 3 to 5 min of sampling. Some dolphins showed no visible reaction to misses or to direct hits. The reactions of these animals were often indistinguishable between hits and misses.

Several studies, regardless of species and size, have noted the absence of strong reactions, thus assuming their method is safe for the animals and the researchers aboard (Jahoda et al., 1996, with fin whales; Gauthier & Sears, 1999, with blue and fin whales; Hooker et al., 2001, with northern bottlenose whales; Best et al., 2005, with southern right whales; Bilgmann et al., 2007, with bottlenose dolphins; Jefferson & Hung, 2008, with Indo-Pacific humpback dolphins; and Cunha, Azevedo & Lailson-Brito Jr., 2010, with Guiana dolphins).

The strong responses that have been reported are usually associated either with the dart not detaching immediately off the animal's body, entanglement of retrieval lines or due to a strong impact, either by power of projector or proximity to the target (Aguilar & Nadal, 1984; Brown, Kraus & Gaskin, 1991; Weinrich et al., 1991, 1992; Gauthier & Sears, 1999; Bearzi, 2000; Krützen et al., 2002; Parsons et al., 2003).

It is important to note that even though this is a minimally invasive procedure it can still pose risks to the animal's health, e.g. high level of disturbance leading to a hazardous stress response. Bearzi (2000) reported the death of a common dolphin, probably as a consequence of either vertebral trauma or acute stress, following the retention of a biopsy dart in the animal's dorsal area, so the precautionary approach should always be favoured.

Previous studies reported that whales that responded to biopsy sampling typically resumed their normal behaviour immediately or a few minutes after the response (Whitehead et al., 1990; Brown et al., 1991, 1994; Weinrich et al., 1991, 1992; Lambertsen et al., 1994; Barrett-Lennard et al., 1996; Gauthier & Sears, 1999). Nonetheless there are exceptions, as Jahoda et al. (2003) noted with fin whales (*Balaenoptera physalus*). When disturbance ceased, these animals' surfacing activity didn't completely return to pre-disruption conditions during one hour of post exposure control and its feeding behaviour got suspended.

Biopsy sampling wounds usually heal quickly with no reported physiological complications (Weller et al., 1997; Krützen et al., 2002; Parsons et al., 2003; Jefferson & Hung, 2008; Giménez, Stephanis, Gauffier, Esteban & Verborgh., 2011; Tezanos-Pinto & Baker, 2012).

Several studies on *Tursiops* spp. have shown the wounds produced by the darting tend to be covered with tissue by approximately a month, ranging from 18 to 42 days (Weller et al., 1997; Parsons et al., 2003; Krützen et al., 2002; Fruet et al., 2017). Jefferson and Hung (2008) biopsied Indo-Pacific humpback dolphins (*Sousa chinensis*) that showed even earlier healing, under 21 days. Giménez et al. (2011) biopsied long-finned pilot whales (*Globicephala melas*) and showed how the wounds closed as fast as four days and that total recovery of the site, entailing re-pigmentation, took place in less than a year (approximately 260 days).

7 - Behavioural reaction to biopsy sampling by non-target animals

The behavioural responses to biopsy sampling by nontargeted dolphins have been poorly studied. Barret-Lennard et al. (1996) and Weller et al. (1997) were the first to note the group's reaction although no thorough assessment was made on these affiliates. Only more than a decade later the first real attempt at assessing the group's reaction to the biopsy procedure was published. In 2008, Gorgone and colleagues recorded the number of nontarget animals reacting as well as their distance to the target animal. They determined the probability of a nontarget animal reacting to biopsy darting decreased with increasing distance from the target animal, which was expected, but that it also decreased with increased sea state. This led them to believe that dolphins may be responding to visual cues and thus with a harshened sea state there would be an increment in water turbidity, preventing the animal to perceive both the reaction of the target dolphin as well as visually identify the dart hitting the water. It was also interesting to find out groups tend to react more if at deeper waters and that the probability of a reaction decreased during summer. They considered the possibility of the animals being more habituated to ambient noise in the summer (rather than in the winter) due to a high degree of vessel traffic (dolphin-watching tours) during that particular season.

Weller et al. (1997) and Mann (1999), noted how the social character of dolphins influences their behaviour to stimuli. Therefore, behaviours of different individuals within a group are likely dependent on one another, which means that the response of nontarget animals might be conditioned by their sampled affiliate. It would only make sense we start evaluating group reaction the same way we do for individual reaction to assess further dependency. Later, Tezanos-Pinto and Baker (2012) decided to classify the group behaviour according to six categories, the same used for individual reactions they analysed from the target dolphins. In both their studied populations of bottlenose dolphins (*Tursiops truncatus*), the groups' responses were mainly mild, proving once again the low impact of such research tool on the overall behaviour and welfare of the species.

8 - Behavioural reactions to misses

Weinrich et al. (1991) published their results on projectile darts being used on humpback whales (*Megaptera novaeangliae*) to collect skin biopsies. In this assessment they noted the whales also reacted to biopsy attempts that failed to acquire tissue samples and to missed shots. They concluded there was no difference in response to hits, regardless a sample being acquired, but that there was a significant difference in the intensity of the behavioural reaction between hits and misses, with the first one naturally eliciting more intense reactions. They assumed it was likely that some portion of the immediate response resulted from stimuli other than the contact from the dart itself, and that the close approach of the vessel or the splash caused by the dart hitting the water might had been perceived by the animal and contributed to its response.

Clapham and Mattila (1993) mentioned whales responded in similar ways to many misses suggesting that a large component of any reaction (hit or miss) is a startle response, meaning whales may just show surprise to a sudden stimulus (whether tactile in the case of a hit, or auditory in case of a miss). More recently, Krützen et al. (2002) described how the bottlenose dolphins in Australia and Brazil reacted the same level and frequency to hits and misses. A similar conclusion was achieved by Jefferson and Hung (2008), suggesting the animal's response to either hit or miss situations appeared to be a startle reaction instead of a response to any pain caused by the impact or penetration of the biopsy dart. Reaction to misses was found at a rate of 12.2% (Clapham & Mattila, 1993) and 16% (Brown et al., 1994) in humpback whales, 55% in sperm whales (Whitehead et al., 1990), 20% in bottlenose whales (Hooker et al., 2001), and 73.7% in other populations of bottlenose dolphins (Krützen et al., 2002).

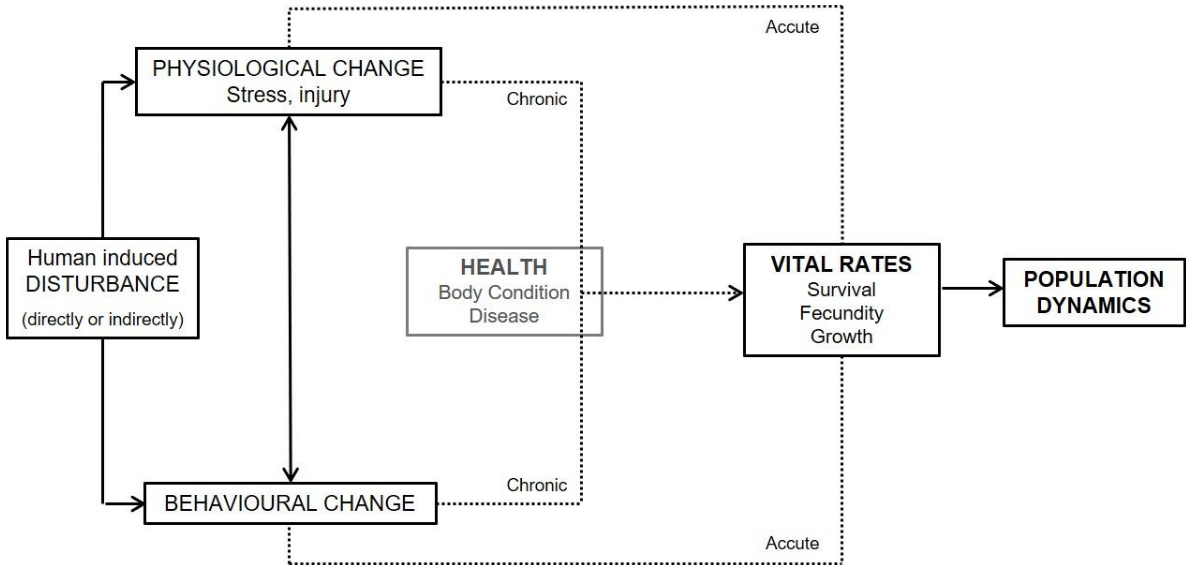
In resume, a reaction to a miss/unsuccessful hit may indicate the animals are also reacting to an external stimulus other than to the physical contact with the dart. The disturbance might be caused by the arrow/dart striking the water (sound and visual provocation). It is important to add that, even though marine mammal researchers try their best to evaluate the animals' behaviour response directly associated to the biopsy technique, there might still exist other external factors that affect it and to which the researchers might be unaware of (Noren & Mocklin, 2012). For example, there may exist unintentional and unobserved behavioural effects on dolphins over the close approach of the vessel necessary to the procedure (Jahoda et al., 2003; Lusseau, 2006; Gorgone et al., 2008), therefore a careful approach should always be taken. Possibly the disruptive effects can be reduced by approaching the group parallel to their direction of travel (Barrett-Lennard et al., 1996, Krützen et al., 2002). However, and despite the source of disturbance, most reported behavioural reactions during biopsy procedures appear to be inconsequential and similar to those observed during whale-watching activities (Noren & Mocklin, 2012).

9 - Long-term behavioural impacts

Stress responses are key to survival. They allow the animal to acknowledge a disturbance and its effects on its homeostasis and to respond to it, whereas through a behavioural shift or a physiological adjustment. However, excessive or continuous disturbance may greatly affect the animal's fitness (Acevedo-Whitehouse & Duffus, 2009). Each individual has its own energetic budget on which they rely to decide which activities are required to spend more or less time on, such as feeding *versus* mating. It is a dynamic and daily adjusted budget with the ultimate goal of surviving and thriving. When there is a stress, the added energetic costs of maintaining a functional immune system will reflect on the remaining physiological processes, like reproduction, growth and development. Additionally, in the eventuality those needs aren't met it is likely the incidence of disease, which can bring animals to a poor condition and increase chances of mortality (Acevedo-Whitehouse & Duffus, 2009). Therefore, this accumulation of energetic challenges that may lead to overall reduced individual fitness, has the potential for, at least from a theoretical perspective, influence the population's viability (Lusseau & Bejder, 2007).

Behavioural changes to disturbance are varied, from increased levels of vigilance, and decreased resting time, to impairment of mother-infant relationships, reduced foraging/feeding rates, or even the time spent in courtship and mating. All of which can potential affect survival, fecundity or growth (Gill, Norris & Sutherland, 2001). Avoidance and displacement are other probable behavioural responses due to disturbance. Biopsy sampling although considered a minimally invasive procedure, requires a research vessel approximation, causing increase disruption. It was commonly accepted that species who actively avoid vessels or which react too strongly to a boat approximation were the ones needing extra care (Bedjer, Samuels, Whitehead & Gales, 2006). However Gill et al. (2001) brought to attention how avoidance is a multifactorial decision. Animals' ranges are determined by local habitat quality and distance to other possible suitable sites, predation risk, competition degree and investment already put into that area it inhabits, like fights for territory or habitat surveying to access its potential. To abandon that area, even when under constant disturbance, requires careful consideration. Dolphins with several alternative habitats may avoid disturbance even when this one is light, whereas animal with little site opportunities can't "afford" to avoid the disturbance, which in turn means they will constantly be under stress, leading to reduced fitness (Lusseau & Bedger, 2007). It is generally accepted that if the ability of individuals to survive, reproduce or grow is affected by anthropogenic disturbances, the conservation status of the whole population may be at risk (King et al. 2015; Figure 10). However, there is a need to quantify how such changes may truly impact individual fitness, and only then we will be able to estimate population dynamics effects more confidently (Lusseau & Bejder, 2007).

Figure 10 - Modelling population level consequences of disturbance. Modified from King et al., 2015.



Assuming that biopsy sampling results mostly in mild short-term reactions from the sampled animals, it is expected to not compromise the population's survival and health nor induce displacement and vessel avoidance. In order to be able to prove such assumptions long-term monitoring is required.

Long-term studies are difficult to come by since they are time consuming and expensive. There are just a few authors that have attempted to determine the long-term effects of biopsy sampling on free-ranging cetaceans. Scientists rely on resighting rates to estimate whether or not the individuals targeted abandon the area or keep inhabiting it. Weinrich et al. (1991) sampled humpback whales and estimated no long-term adverse responses were present, such as individual or herd displacement, based on their high resight rate over the years the study took place. The same year the International Whaling Commission stated biopsy sampling of cetaceans wasn't likely to produce any long-term deleterious consequences. Although recommended for more long-term studies to be taken, given thorough information was lacking. Recently, Tezanos-Pinto and Baker (2012) compared resight rates of dolphins before and after sampling and found no statistical significance, meaning the dolphins kept using the area after being sampled. Furthermore, they determined the probabilities of capturing biopsy sampled versus non-biopsy sampled animals were similar thus concluding there were no repercussions on long-term residency patterns. They also noticed that biopsied animals kept approaching the research boat and allowing for photo-identification, just like Krützen et al. (2002) had previously suggested. Curiously, they weren't the first, several studies report that biopsied animals don't seem to avoid vessels during subsequent approaches (humpback cow/calf pairs, Weinrich et al., 1991; killer whales, Barrett-Lennard et al., 1996; bottlenose dolphins, Hooker et al., 2001; Indo-Pacific humpback dolphins, Jefferson & Hung, 2008).

Many authors have reported that animals that responded to biopsy sampling typically resumed their normal behaviour immediately or a few minutes after the response (Whitehead et al., 1990; Brown et al., 1991, 1994; Weinrich et al., 1991, 1992; Lambertsen et al., 1994; Barrett-Lennard et al., 1996; Marsili & Focardi, 1996; Gauthier & Sears, 1999; Hooker et al., 2001, Fossi et al., 2003).

Moreover, there were reports from both habituation and sensitization of cetaceans to repeated biopsy attempts. Gauthier & Sears (1999) studied 4 balaenopterid whales (blue, fin, humpback and minke whales), and stated that repetitive attempts seemed to incite lower intensity reactions. In contrast, Best et al. (2005) noted how female southern right whales with calves would increase intensity of reaction to repeated biopsy sampling. Although there seems to be a stronger reaction from females with calves, these examples show how conclusive studies on habituation and sensitization are still lacking.

In sum, there seems to be no indications of any long-term effects, such as avoidance of the sampling area (sperm whales, Whitehead et al.,1990; humpback whales, Weinrich et al., 1991, Clapham & Mattila, 1993; killer whales, Barrett-Lennard et al., 1996; bottlenose dolphins, Weller et al., 1997, Tezanos-Pinto & Baker, 2012; Indo-Pacific humpback dolphins, Jefferson & Hung, 2008). Although with low statistical power, Best et al. (2005) also did not find any adverse effects on the reproductive cycles and calf survival. Presently, there is still a lack of empirical evidence to allow to quantify the causal relationship between behavioural/physiological changes and fitness (King et al., 2015). Nonetheless, given the sparse research on the subject and attending the potential underestimation of detrimental population effects, the precautionary approach should be preferred. This includes, improving research techniques that would decrease the required contact period, avoiding sampling groups with infants and continuing and expanding this kind of response analysis studies.

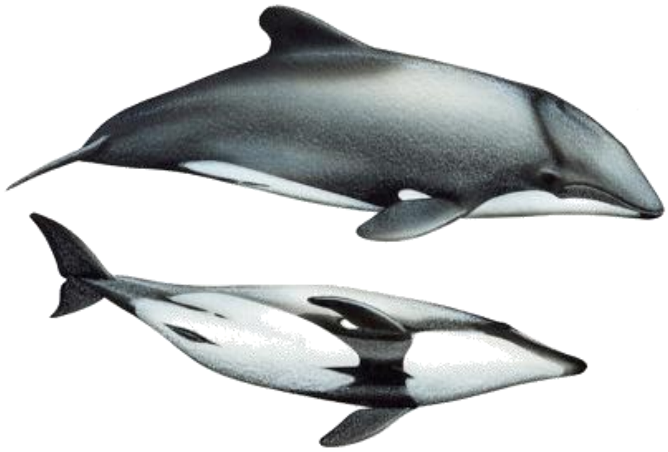
10 - Cetaceans off the Chilean coast

The Chilean and Peale's dolphins are among the least studied species of the dolphin family (Viddi, Harcourt, Hucke-Gaete, & Field, 2011). They are coastal species, the first restricted to the waters of Chile and the second to both Chile and Argentina, and whose distribution greatly overlaps with human activities.

10.1 – Chilean dolphin

Scientific name: *Cephalorhynchus eutropia***,** Gray 1846.

Figure 11 - *Cephalorhynchus eutropia* © Wurtz-Artescienza.



10.1.1 - Description

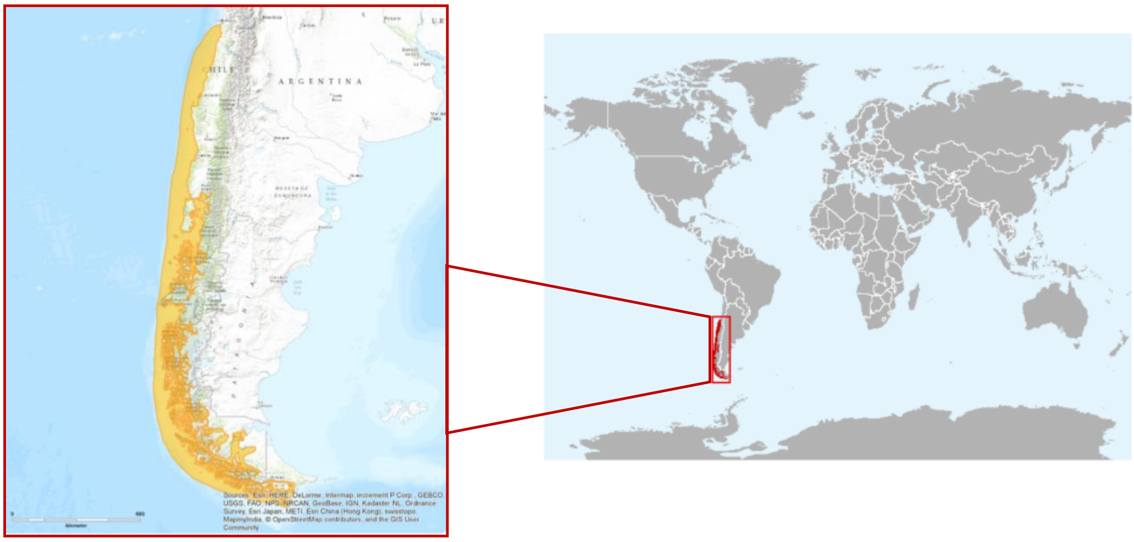
The Chilean dolphin (Figure 11) belongs to the genus *Cephalorhynchus*, to which four strictly coastal species that live in cool temperature latitudes over the South Hemisphere belong. This species is the only endemic cetacean of the coastal waters of Chile (Goodall, Norris, Galeazzi, Oporto, & Cameron, 1988). Like all members of *Cephalorhynchus* they are small, chunky and blunt-headed animals with no discernible beak, ranging from 123 to 167 cm (Goodall, et al., 1988). Its body weight can vary between 30 and 62 kg (Oporto, Brieva, & Escare 1990; Dawson, 2009). The dorsal fin is proportionally large and with a rounded convex edge (Dawson, 2009).

Their overall appearance is dark grey, with a dark semilunate mark behind the blowhole (Heinrich, 2006) making the melon appear in a lighter shade of grey. Their throat and belly are white with a dark grey band connecting the flippers, a dark caudal peduncle and a dark genital patch with sex-specific pattern (Heinrich, 2006).

10.1.2 - Distribution and ecology

The Chilean dolphin is restricted to cold, shallow, coastal waters of southwestern South America, whose habitat (Figure 12) ranges from Concón (32°56'S) near Valparaiso to Navarino Island (55°14'S) close to Cape Horn (Goodall, 1994; Aguayo-Lobo, Torres & Acevedo, 1998). Its distribution appears to be continuous, and yet it seems to exist areas of local abundance, such as off Playa Frailes, Valdivia, Golfo de Arauco, and around Isla de Chiloé (Dawson, 2009).

Figure 12 - Distribution of *Cephalorhynchus eutropia*: coastal waters of Chile and southern Argentina (Heinrich & Reeves, 2017; © IUCN).



As stated by Goodall (1994) it is possible to distinguish two different types of habitat: 1) the open coast, bays and estuaries north of Chiloé, such as waters near Valdivia and Concepción; 2) the channels, fjords and protected waters between Cape Horn and Isla Chiloé. Primarily seem to prefer productive shallow waters, usually sheltered and close to shore, with rapid tidal flow and input from rivers (Heinrich, 2006). The species is known to enter rivers (Carwardine, 1995) and estuaries (Goodall et al., 1988, Goodall, 1994; Carwardine, 1995).

It appears their movements are quite limited and that their areas of residence are small and restricted (Heinrich, 2006; Viddi pers. comm., April 2007), hence the importance of assessing the impact of anthropogenic activities on their ecology.

Carwardine (1995) suggested that the animals, in the southern part of the range, tend to be more suspicious of boats and hard to approach, whereas in the north, they have been identified swimming over to boats and even bow-riding.

Groups tend to be small, generally between 2 and 10 individuals (Goodall et al., 1988; Heinrich, 2006), although most observers have reported sighting only two or three animals at each time. Relatively large aggregations (20-50) have also been described (Goodall, 1994; Pérez-Álvarez, Alvarez, Aguayo-Lobo, Olavarría, 2007), which may represent occasional aggregations of smaller groups (Culik, 2010a).

The Chilean dolphins are fully sympatric with Peale's dolphins (Heinrich, 2006). Although mixed groups of Chilean and Peale's dolphins have been observed, a clear pattern of spatial and temporal partitioning of coastal habitat by the two species was documented during a six-year study at Isla Grande de Chiloé (Heinrich, 2006). This pattern might not apply in other areas, such as farther south in the Guaitecas Archipelago, where mixed groups are often observed foraging and socializing (Viddi pers. comm., April 2007).

10.2 – Peale's dolphin

Scientific name: *Lagenorhynchus australis,* Peale 1848.

Figure 13 - *Lagenorhynchus australis* © Würtz-Artescienza

10.2.1 - Description

The Peale's dolphin (Figure 13) belongs to the genus *Lagenorhynchus* (*Delphinidae, Cetacea*), along with 5 other species (three from the southern hemisphere and the other three from the northern one).

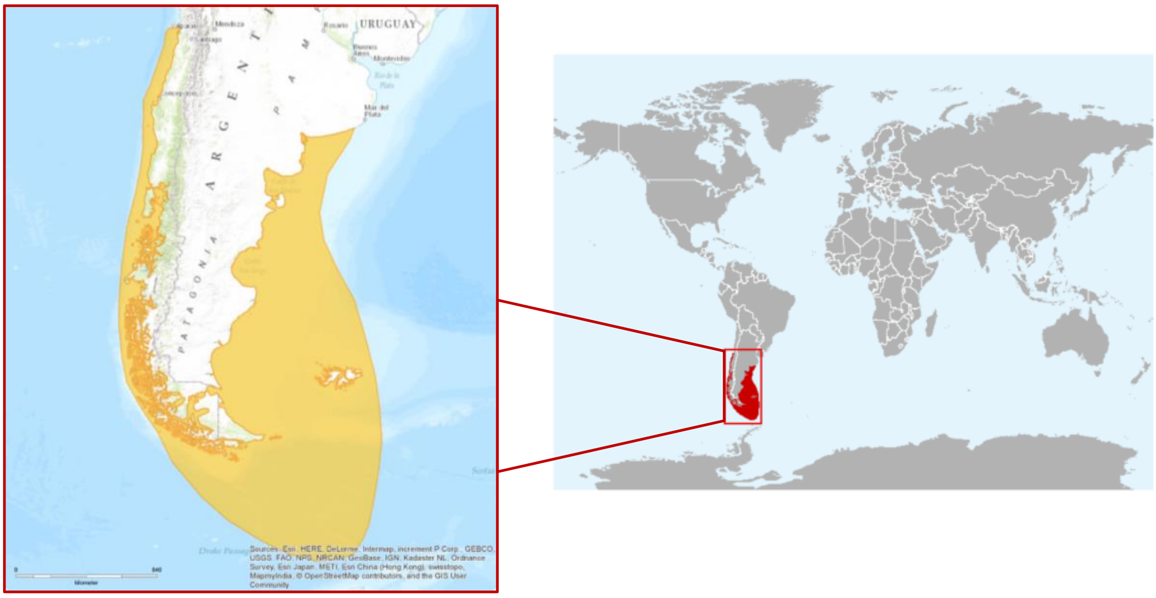
The *L. australis* is a stocky dolphin with an unobtrusive beak. Its length reaches 210 cm in females and 218 cm in males; and the heaviest animal weighed 115 kg (Goodall, Norris, et al., 1997). Its colour is dark grey or black on the dorsal side, with two areas of lighter shading on the flanks (Heinrich, 2006). As the Chilean dolphin it also has distinctive white axillary marks. The larger thoracic patch is light to medium grey, outlined with a narrow dark line on its lower surface. Their dorsal fin is falcated.

10.2.2 - Distribution and ecology

It is the species with the most limited range from the *Lagenorhynchus* genus, being restricted to the coastal waters of southern South America (Figure 14), comprising Chile, Argentina and the Falkland Islands (Brownell, Crespo & Donahue, 1999).

It ranges along the Chilean coast from Valparaiso (33°S) southward to Tierra del Fuego (59ºS) and along the Argentinean coast northward up to San Matias Gulf (38°S) (Goodall, de Haro, Fraga, Iñiguez & Norris, 1997; Brownell et al., 1999; Goodall, 2002; Viddi et al., 2011). This species is also found around the Falkland Islands (Webber & Leatherwood, 1991; Aguayo-Lobo et al., 1998).

Figure 14 - Distribution of *Lagenorhynchus australis*: coastal waters of Chile and southern Argentina (Hammond et al., 2008; © IUCN).



The Peale’s dolphin is the most coastal, and therefore the easiest to observe of the *Lagenorhynchus* genus species (Viddi & Lescrauwaet 2005). They occupy two major habitats: open, wave-washed coasts over shallow continental shelves to the north; and deep, protected bays and channels to the south and west. In the channels, this is an 'entrance animal', associated with the rocky coasts and riptides at the entrance to fjords (Hammond et al., 2008). Although they have been observed in waters at least 300m deep, they appear to prefer shallower coastal waters (Brownell et al., 1999 and refs. therein).

Peale's dolphins show a high degree of association with kelp beds (*Macrocystis pyrifera*), especially in the channel regions. They swim and feed within, inshore and offshore of the kelp forests, using natural channels for movement (Goodall, de Haro, et al., 1997; de Haro & Iñiguez, 1997).

Peale's dolphin is known to ride bow-waves of large vessels and may swim alongside smaller ones (Culik, 2010b). These animals are usually seen in small groups of 2 to 20 individuals and may associate with Risso's and Commerson's dolphins (Jefferson, Leatherwood & Webb., 1993; Brownell et al., 1999 and refs. therein).

From some stomachs that were examined it is thought this dolphin species feeds on molluscs, crustaceans (shrimp), cephalopods (octopus and squid), and fish (kingklip fish, hagfish and southern cod) that occur in shallow waters and amongst kelp beds (Brownell et al., 1999).

10.3 - Threats faced by the two dolphin species

Direct Catch:

Since 1977, the hunting of dolphins has been considered illegal (Supreme decree nº 381, Chile) although poorly regulated or supervised. While the killing of dolphins was prohibited by law, there are several reports of its use as bait in ring nets for crustaceans (particularly for the lucrative southern king crab, *Lithodes santolla*, and false king crab, *Paralomis granubosa*), in longlines targeting sea bass (*Eleginops maclovinus*) and in individual hooks targeting swordfish (*Xiphias gladius*) (Aguayo-Lobo, 1975; Goodal et al., 1988; Lescrauwaet & Gibbons, 1994; Carwardine, 1995).

Direct hunt with harpoons is possible given the dolphins' social behaviour of approaching the fishing boats. It's estimated that in the 1980s 1300 to 1500 dolphins were harpooned each year. In 1992, Aguayo-Lobo et al. reported the death of approximately 600 dolphins for bait use, and two years later Goodall (1994) noted that the captures for bait continued. Sielfeld (1983) added that in the south area of the Magellan region it had been hunted for human consumption at the oil exploration platforms. No recent estimates are available on the number of marine mammals killed for bait (Brownell et al., 1999), but it is thought to be lower than in the past (Goodall 2002) or even to have ceased. This is believed to be correlated with the decrease in dolphin numbers, fisheries and in the king crab stock (Aguayo-Lobo et al. 1998) and the development of cheap alternative bait sources (Lescrauwaet & Gibbons, 1994).

Incidental catch:

There are a few studies that reported the frequency on which these species would get caught and drowned in different types of nets (Cárdenas, Oporto & Stutzin, 1986; Goodall, et al., 1988; Jefferson et al. 1993; Reyes & Oporto, 1994; Lescrauwaet & Gibbons, 1994, Aguayo-Lobo, 1999; Brownell et al., 1999).

Incidental catch of the Chilean dolphin (Figure 15) occurs possibly throughout its range, especially in the north. No true calculation has been made of the extent of incidental catch in Chile, but Goodall (1994) implied that approximately 70 animals are caught per year at the port of Queule, south of Valdivia. This transposed to a country scale would represent a far bigger number. An unknown number of Chilean dolphins are also caught in shore-based gillnets (Figure 16) set by local people from Isla Chiloé to capture small native fish and introduced farmed salmon that have escaped from their cages (Heinrich, 2006).

Regarding the Peale's dolphin, while in the northern part of its Pacific range there are seldom dolphins taken in gillnets (Goodall, 2002), around the Chiloé Island there are reports of entanglements in anti-pinniped nets related to salmon aquacultures (Goodall, 2009). Their close dependence on kelp forests may render them vulnerable to habitat loss (Viddi & Lescrauwaet, 2005).

Figure 16 - Chilean dolphin accidentally caught. (photo credit: YaquPacha ©).

Figure 15 - Porpoise caught in a shore-based gillnet. (photo credit: YaquPacha ©).

<u>Aquaculture:</u>

At present, the main conservation concern for coastal small cetaceans in southern Chile is the progressive destruction of critical habitat due to coastal development and intense aquaculture, comprised of salmon and mussel farms (Figure 17). In fact, Chile is the second largest producer of farmed salmon (*Salmo salar* and *Oncorhynchus* sp.), after Norway (Soto & Norambuena, 2004). These farms are heavily concentrated along the coasts in southern Chile, overlapping the habitat of coastal cetacean species and restricting space available for biologically important dolphin behaviours (Heinrich, 2006; Ribeiro, Viddi, Cordeiro & Freitas, 2007). Moreover, this industry is known to use 70 to 300 times more antibiotics than those in Norway (Millanao et al., 2011), a practice which is thought to have substantial adverse effects on the health of native marine animals (Cabello, 2004, Buschmann et al., 2009).

Areas once pristine are now affected by residues and contaminants that result from aquaculture, construction of infrastructure, increase in maritime traffic, and industrial development (Buschmann et al., 2009). Furthermore, the acoustic harassment due to the devices intended to dissuade pinnipeds from predating on fish farms and from increased boat traffic, result in dolphins abandoning the area (Ribeiro, Viddi & Freitas, 2005). There is also evidence that these mammals are sometimes caught incidentally in anti-sea lion nets set up around salmon farms in the fjords and channels (Heinrich & Reeves, 2017).

The limited distribution and relatively inflexible habits of *Cephalorhynchus* spp. makes them particularly vulnerable to fragmentation and population loss in the face of increasing human activities.

Figure 17 - Aerial caption of the mussel farms on Chiloé Island (photo credit: Sonja Heinrich).

Hydropower:

Coastal degradation from industrial and urban development is recognized as the major threat to coastal cetaceans worldwide (Whitehead, Reeves & Tyack, 2000). The close association with riverine and estuarine ecosystems makes Chilean dolphins extremely vulnerable to habitat loss both from coastal and upstream river basin degradation (Viddi, Harcourt & Hucke-Gaete, 2015). Northern Chilean Patagonia is currently being considered as a new frontier for the development of hydroelectric dam projects. This will inevitably affect water flow and nutrient regimes to associated rivers and have profound effects on the complex oceanographic processes that sustain Chilean dolphin critical habitat.

10.4 - Conservation status

The International Union for Conservation of Nature (IUCN) categorized in 2017 the Chilean dolphin as Near Threatened (NT). The best available information indicates that the total population size is only in the low thousands, meaning that the number of mature individuals is likely to be fewer than 10,000 and therefore it is likely that the population size threshold for Vulnerable under criterion C is met. Since no estimate of decline rate neither consistent numbers of population size are available the species is best considered NT (IUCN, 2017).
As for the Peale's dolphin, due to a lack of adequate information to assess the extinction risk for this species (absence of a population estimates, and lack of an assessment of the impact of the use as crab-bait), it was categorized as Data Deficient by the IUCN in 2008 and it hasn't changed yet.

CHAPTER II - Study

1 - Aims of the study

Tissue sampling can always potentially affect welfare, from physical injury to stress and disturbance of the animal's behaviour. As researchers it is our responsibility to try to maintain the welfare of the subject of our studies and it involves choosing the least destructive and painful technique possible, in order to minimise the risks to the animals (de Vere et al., 2018). This preliminary study aims to determine the effect of a novel biopsy sampling protocol on the short-term welfare of two species of small cetaceans (*Cephalorhynchus eutropia* and *Lagenorhynchus australis*) through a behavioural assessment. For this purpose, the sampling attempts were recorded on video (whenever possible), using two waterproof cameras on different angles, and later analysed and classified according to the discomfort/disturbed behaviour shown.

Specific objectives:

1 - Record the short-term behaviour of two species of coastal dolphins (Chilean and Peale's dolphins), before, during and after a skin biopsy collection protocol;

2 - Categorise the immediate behavioural responses of both dolphin species (Chilean and Peale's dolphins) to the biopsy procedure;

3 - Determine the ecological variables that influence the response of the two species of dolphins (Chilean and Peale's dolphins) to the skin biopsy procedure;

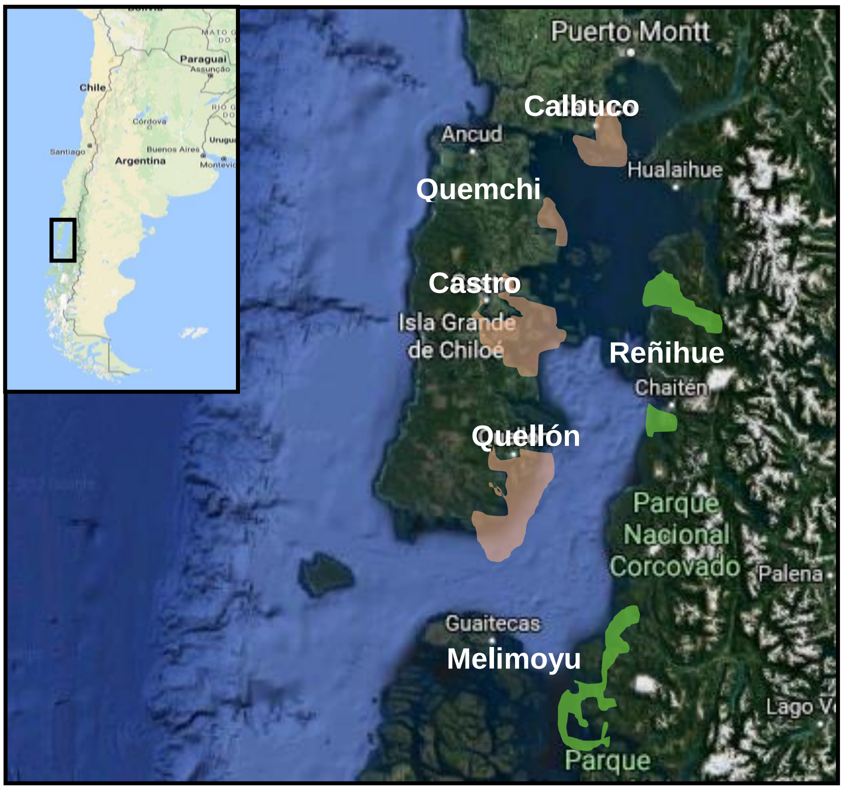
4 - Validate the set protocol as an efficient sampling technique, i.e. that we were able to collect viable and well-sized tissue samples.

2 - Materials and methods

2.1 - Study area

Our data was collected between December and April of 2015, 2016 and 2017, in northern Patagonia, Chile (Figure 18); in an area correspondent to the Chiloense marine ecoregion (41-44ºS, 73-74ºW). This region was divided into 6 different sections: Castro (C), Calbuco (Cb), Quellón (Q), Quemchi (Qm), Melimoyu (M) and Reñihue (R). The first four locations were considered highly disturbed by anthropogenic activities due to the presence of numerous mussel and salmon farms in the area, whereas the last two locations were considered minimally distressed areas.

Figure 18 - North of the Chiloense marine ecoregion. In orange the sites considered disrupted by human activities (Calbuco, Castro, Quellón and Quemchi) and in green the areas considered the closest to the natural primitive habitat (Melimoyu and Reñihue).



The areas around Chiloé Island consist in shallow coastal waters, that form mainly protected bays. As for the sites in the continental coast, they comprise pristine fjords and channels that are characterized by high volumes of freshwater run-off from the Andes Ridge combined with abundant precipitation, making the whole region a vast estuarine system (Silva, Calvete & Sievers, 1997, 1998).

2.2 - Coastal surveys

As part of an on-going health assessment on coastal dolphins, vessel-based surveys were taken during the austral Summer and Fall (January until April). The surveys were conducted aboard of a 4m inflatable boat powered by a 20-horsepower (hp) outboard engine, crewed by a driver-observer, photographer-data recorder and a biopsy sampler-observer (Figure 19). Some days a fourth element joined and helped the observational efforts. A constant survey speed of 12 knots (22.22 km/h) was maintained. Daily routes followed coastal strip transects at about 250m distance from the coast to maximize our chance of spotting dolphins. When spotted, trained observers determined the initial behaviour of the animals and the presence of calves, while breaking off transect to approach the animals for photo-identification.

Figure 19 - The 4 meters inflatable boat with the 20 HP outboard engine, used to perform the biopsy sampling surveys. Photo credits: YaquPacha©.

Once the group of dolphins was approached, we recorded the location (geographical position using Garmin Oregon 400c portable Global Positioning System), time (of the beginning and ending of the sighting and the moment of the biopsy attempt), estimated group size (number of dolphins) and composition (how many calves, juveniles and adults were in the group), predominant behaviour and group cohesiveness. When sighted, the group was tracked throughout the entire observation period until it was lost (group follow protocol, after Mann, 1999).

A group of dolphins (a sighting) was defined as any aggregation of two or more dolphins (including all age classes) which were ≤100m from each other, spaced less than 10 body lengths apart (Heinrich, 2006). The following definitions were established for group composition: neonate (less than 1/3rd of the adult size and showed clear foetal fold marks, being seen in constant affiliation with an adult); calves (up to 1/3rd body length of an adult and without foetal fold marks); juveniles (more than 1/2 but less than 3/4 the body length of an adult); and adults (individuals with approximately 1.67 m long for Chilean dolphins [Dawson, 2009] and for 2.2m for Peale's dolphins [Heinrich, 2006]). Group cohesiveness was defined as either loose (more than one adult body length apart) or tight (less than one adult body length apart) (Viddi & Harcourt, 2016). Group size was categorized as either small (1-4 animals), medium (5-9 animals) and large (≥ 10 animals).

The predominant group behaviour (Table 4) was defined as being the activity in which most animals (>50%) were engaged in and was assigned by a focal group sampling method (Mann, 1999).

Table 4 - Behavioural state definitions (adapted from Shane, Wells & Würsig, 1986; Viddi & Lescrauwaet, 2005; Heinrich, 2006 and Peters, Parra, Skuza & Möller 2013).

Behavioural state	Definition
Chasing (Ch)	Rapid directional surface swimming where dolphins produced splashes and "rooster tails". Individuals surfaced synchronously and moved rapidly in one or several offset lines, usually parallel to the shore.
Feeding (F)	Dolphins involved in any effort to capture and consume prey as evidenced by chasing on the surface, deep diving and circle swimming. Repeated unsynchronised dives in different directions in a specific location. Prey might be observed sometimes as well as seabirds "plunge diving" or consuming prey in the same location.
Milling (M)	Dolphins show frequent changes in heading but with no net displacement. Regular dives of varying duration. Probably represents them opportunistically scanning and searching for food but presence of prey could not be confirmed.
Resting (R)	Dolphins engaged in very slow movements or stationary at the surface, often seen floating motionless at the surface, interspersed with slow rolling surfacings.
Socializing (S)	Individual interactions within a tightly aggregated group, characterized by high levels of surface activity: frequent physical contact, often with vigorous movements and aerial behaviours such as leaping and breaching. Sexual and aggressive behaviours are included in this category.
Traveling (T)	Dolphins engaged in directional and persistent movement at constant speed with regular surfaces. No splashes or abrupt movements and no prolonged dives.

A number of abiotic data were also noted: sea state (Beaufort wind scale, appendix 1), cloud coverage (% estimated), depth, sea surface temperature and salinity, distance to shore and anthropogenic sites, such as fish and mussel farms (determined by a laser range finder), visibility of the water (using a Secchi disk with a 30cm diameter) and presence or absence of rivers and kelp forests (important variables for habitat selection in Chilean and Peale's dolphins, respectively).

2.3 - Biopsy sampling collection

Once approaching the animals, a constant speed was maintained, and the boat was maneuvered to travel parallel to the dolphins to minimize disturbance (Gorgone et al., 2008). Sampling attempts began only after all individual dolphins within the group had been photographed (Durban, Parsons, Claridge, & Balcomb, 2000) and the target animal identified. We scanned the animal for previous sampling mark to prevent resampling the same individual. Other marks as pigmentation defects, fin notches, and scars, were recorded in order to avoid repeating the sampling in the future (Bilgmann et al., 2007).

All assistants were trained before entering the field and everyone's responsibilities and tasks on board were clearly defined. Only experienced scientists performed the procedure of biopsy sampling. Before using any biopsy system on live animals, tests on land first and later at sea were conducted in order to refine its application on a moving target, while avoiding the risk of unnecessary harm to the animals (Patenaude & White, 1995).

All the biopsies were collected by the same researcher that would always ride at the bow, to avoid possible errors by variating the way the technique was applied. So that we could properly appreciate the dolphin movements, the procedure took place only in a sea state less than Beaufort 3 (Appendix 1) and during daylight hours.

A 1.7m Hawaiian spear was adapted to hold a biopsy headpiece (Figure 20). This tip was a stainless-steel cylindrical punch, 18 mm long, with cutting edges and three internal barbs, designed to retain a 4 mm in diameter sample (Figure 21). A circular disc (or "stop") around the base of the tip prevents penetration beyond the blubber (18 mm deep) and was responsible for the dart bouncing after sample acquisition (Aguilar & Nadal, 1984; Lambertsen, 1987; Hoelzel & Amos, 1988; Amos & Hoelzel, 1990; Krützen et al., 2002; Cunha et al., 2010).

In order to prevent the pole for sinking fast, in the case it would fall on the water after a biopsy attempt, we created a gross flotation system that consisted on a cylinder piece of foam secured to the opposite end of the pole from where the biopsy headpiece was fixed. This device would give us a few more seconds to a minute to grab a hold of the pole.

Animals were sampled once close to the water surface or when they breached. The procedure was only applied on juveniles or adult individuals. Attending to the skin sensitivity map discussed in the literature review, we knew the importance of selecting the biopsy hit location. Since intruding excessively and causing harm would be our least wanted outcome, choosing a less sensitive area of the body would be ideal. As such we always tried to direct the biopsy sampling attempts at the dorsal-lateral region directly below, and extending posterior to, the dorsal fin (Parsons et al., 2003). All samples were taken under the biopsy permit from the Chilean Under Fisheries RES 3369/2014 and RES 1196/15.

Figure 20 - Pole spear adapted with a biopsy head. The force employed was made by the elastic band.

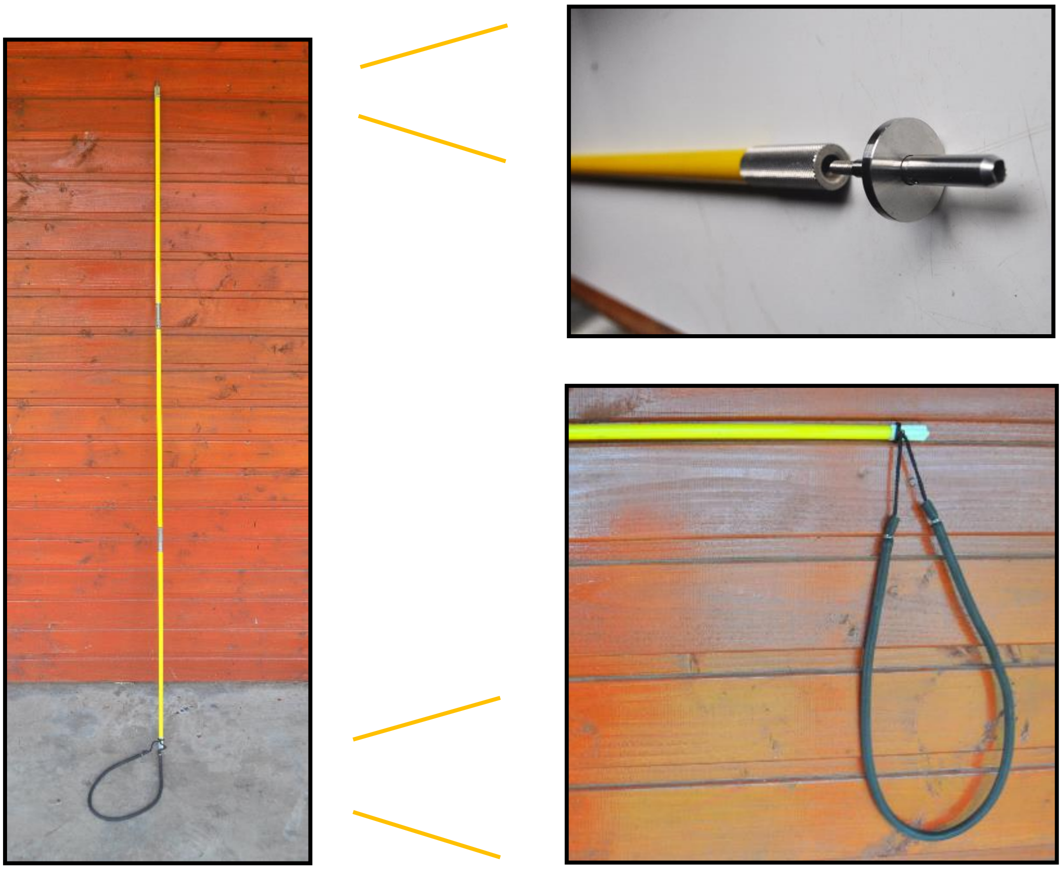
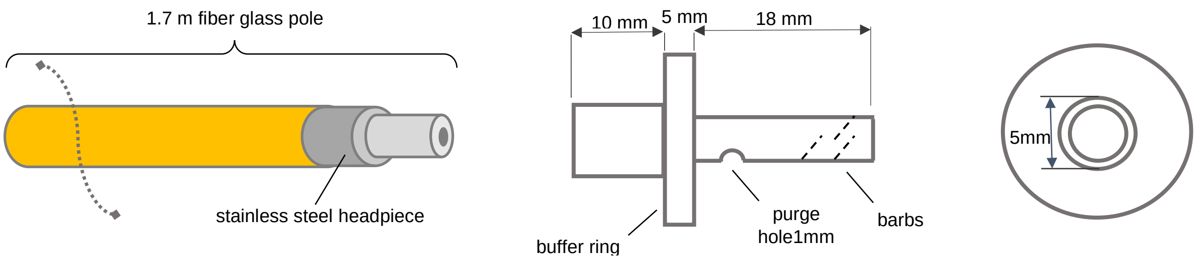


Figure 21 - Detailed schematic of the biopsy tip.



A biopsy attempt was successful whenever having collected a biopsy sample (a positive "hit") or unsuccessful whenever the pole hit the animal, but no biopsy sample was collected (a negative "hit") or when the pole was thrown into the water and didn't hit the dolphin (a "miss"). Once a sample was taken, wearing latex gloves, we extracted it from the biopsy tip by unscrewing the headpiece from the pole and, using sterilised forceps, pushed the sample onto the Petri dish. Samples were then cut into four pieces, each one stored in a different tube: one containing 96% ethanol and another with 10% formalin, both for DNA extraction, and a third tube containing RNAlater for RNA analysis.

They were all kept in a cooler until we got to land, where everything was stored in a freezer at -18ºC. The fourth piece was kept in a tissue cassette inside a flask on 10% formaldehyde, at room temperature, to proceed to histopathology analysis (Figure 22). If more than one successful attempt had been made, the protocol was completed for each sample at a time, followed by a change in gloves and equipment to process the consecutive sample, and so on. All samples were properly identified with a code, which consisted on the initial letter that corresponded to the area from which the biopsy resulted (Calbuco, Castro, Melimoyu, Quellón, Quemchi or Reñihue), followed by the initials of the species Latin name (Ce for the Chilean dolphin and La for the Peale's dolphin). Overall it looked like this: "QLa06" when the sixth Peale's dolphin got sampled in Quellón; or "MCe02" when the second Chilean dolphin's sample was obtained in Melimoyu.

Only when the field season ended the collection of samples acquired were taken into the laboratory to be fully processed and analysed.

Figure 22 - Demonstration of the sample processing a board.

2.4 - Material preparation and sterilization

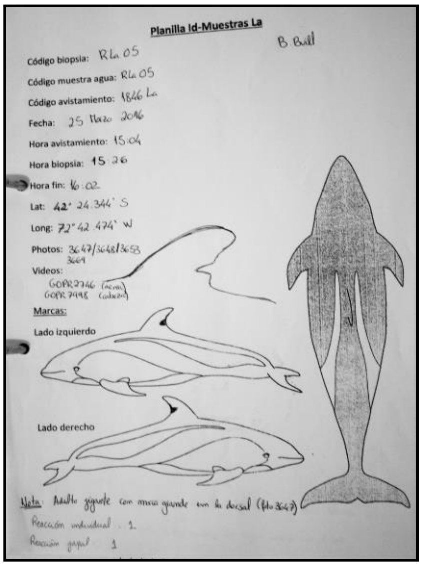
Every day, after the boat surveys were completed and we returned to land, all of the material used on board (waterproof uniform, cases that contained cameras and other important devices, laboratory material, oars, gas tank and anchor) was taken out of the zodiac, rinsed with fresh water and left to dry in the shade. The cameras, lenses, laser distance measurer, salinometer and thermometer were cleaned with a humid cloth to prevent salt from damaging its function.

To remove all tissue residues from the equipment used to process the skin biopsy samples we sprayed chlorine over it, following a scrub done individually using a dish scrubber (used solely for that purpose) with commercial detergent (adapted from Sinclair et al., 2015). The biopsy heads and adapters, surgical tweezers and scissors had to be thoroughly cleaned, and for that we used disposable interdental toothbrushes. Afterwards they were rinsed with fresh water and left to dry at room temperature. All the cleaning procedures were done wearing latex gloves and surgical mask to prevent biological contamination.

In order to prevent contamination of the material on board, we would separate it into "field kits" using a porous paper and tape to seal them. Once the wraps were made, they were set on an autoclave (121º for 15 min). Each kit contained a pair of scissors, tweezers and a surgical stylet. Biopsy heads were wrapped individually and separated from these kits. The same was done for the adapters of the biopsy tips to the pole. The tool box was cleaned with 70% alcohol each day before placing in the new field kits. The latex gloves and any other waste was disposed appropriately.

2.5 - Assessing behavioural reaction to biopsy sampling

Figure 23 - Example of one of our record sheets for the Peale's dolphin. Note the notch on the animal's dorsal fin here recorded.


Planilla Id-Muestras La
B Bull
Código biopsia: RLa 05
Código muestra agua: RLa 05
Código avistamiento: 1846 La
Fecha: 25 Marzo 2016
Hora avistamiento: 15:04
Hora biopsia: 15:26
Hora fin: 16:02
Lat: 42° 24.344' S
Long: 72° 42.474' W
Photos: 3647/3648/3653 3669
Videos:
GOPR2746
GOPR 7998
Marcas:
Lado izquierdo
Lado derecho
Nota:
Reacción individual 1
Reacción grupal 1

Dolphins are hard to follow, diving at uncertain times and without leaving any long-lasting traces. Researchers try to overcome these challenges through the development of photo-identification and the recording of natural markings on the animal's body (generally on the dorsal fin). Being able to identify individuals allows scientists to link sightings from different years or locations (Mann, 1999). In our situation, it allowed us to prevent biopsy sampling the same individual twice. We would not only photograph the individual sampled and record its location (these species have a high degree of site-fidelity), but also note its particular body marks, such as possible wound scars, pigmentation patterns and nicks and notches of the dorsal fin (Figure 23).The behavioural reactions of the targeted animal and the dolphins next to it (non-targeted animals) as well as the outcome of every biopsy attempt were noted after the procedure. We considered that a dolphin responded to biopsy sampling when it immediately modified its behaviour observed before the biopsy attempt. To this effect we used the focal-animal sampling method (Altmann, 1974).

Besides observational effort to determine the animal's behavioural response, we also had video support. The biopsy sampler used a waterproof video camera (GoPro2®) on his head, mimicking his view point of the whole procedure and another crew member (either the driver or the photographer) held a pool pole with another waterproof camera attached at its top end (Figure 24).

The response of the dolphins in relation to the biopsy attempt was classified into five categories according to defined criteria (Table 5).

Figure 24 - Video angle obtained from the camera attached to the pool pole. Arrow indicates the 2nd camera that was secured to the biopsy sampler's head.

Table 5 - Classification of individual and group behavioural reactions of Chilean and Peale's dolphins to biopsy sampling (adapted from Krützen et al., 2002; Bilgmann et al., 2007; Tezanos-Pinto & Baker, 2012; Loizaga de Castro et al., 2013).

Level	Individual behavioural reactions	Group behavioural reactions	Category
0	No noticeable reaction and the targeted individual continues in the vicinity of the boat	No noticeable reaction and the group continues in the vicinity of the boat	None
1	Individual targeted flinches but continues in the vicinity of the boat	Startle reaction; dolphin close to targeted animal flinches, moving with/without speed burst but continues in the vicinity of the boat	Startle
2	Dolphin leaves the vicinity of the boat, with or without underwater acceleration	Dolphin next to targeted animal accelerates underwater and leaves the vicinity of the boat	Mild
3	Single leap or porpoising by the targeted individual that then accelerates, leaving the boat vicinity	Dolphin next to targeted animal accelerates and leaves the vicinity of the boat followed by single leap or porpoise	Moderate
4	Multiple leaps and/or porpoises with splashing by the targeted individual, that then accelerates leaving the boat vicinity	Dolphin next to targeted animal accelerates and leaves the vicinity of the boat followed by multiple leaps and/or porpoises	Strenuous

As a result of video recording the totality of the behavioural reaction, from the moment we started the approach to after the sample was taken, we were able to understand the surface behaviour of both species during the procedure, which will be further presented in the results (Figures 26 - 29).

2.6 - Statistical analyses

All of our data was analysed using RStudio® version 3.4.3 (https://www.R-project.org). For statistical analysis we separated our abiotic factors and other variables according to their nature (Table 6).

Table 6 - List of categorical and numerical variables selected for our study, each of their levels and their type of classification. Underlined are the names our variables were identified as.

Variable		Levels	Type
Categorical	Area	Calbuco; Castro; Melimoyu; Quemchi; Quellón; Reñihue	Nominal
	Behaviour	Chasing; Feeding; Milling; Socializing; Travelling; Resting	
	Dispersion	Tight; Mixed	
	Geography	Continental; Insular	
	GR	0 none; 1 startle; 2 mild; 3 moderate; 4 strenuous	
	IR	0 none; 1 startle; 2 mild; 3 moderate; 4 strenuous	
	Month	January; February; March; April	
	Season	Summer; Fall	
	Size	Small; Medium; Large	
	Species	Chilean dolphin; Peale’s dolphin	
	State	Natural; Disturbed	
	Year	2015; 2016; 2017	
Numerical	Beaufort scale	[0,3]	Discrete
	Depth		Continuous
	Distance to Human activities		
	Encounter Duration		
	Prebiopsy		
	Shore Distance		

We did a descriptive statistical analysis on the numerical independent variables, through the calculation of their mean and standard deviation (Table 8).

Recognizing our data wasn’t normally distributed we chose non-parametric statistical tests for data analysis. The defined significance level for all statistical analyses was $\alpha=0.05$. Kruskal-Wallis tests were performed to compare all numerical variables to our individual and group reactions recorded. Whenever a positive result was found, a post-hoc test (Dunn’s test) was done to further assess dependency.

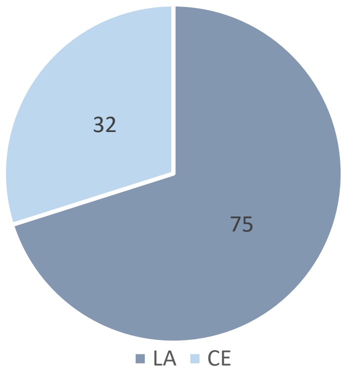
For the nominal variables, instead of using the Chi-square test for comparisons, given our small sample size and several expected cells resulting in a value inferior to 5, we resorted to Fisher’s exact test (Appendix 2). We used it to compare behavioural responses (both individual and group reactions) in relation to all the remaining categorical variables. The frequency table of the categorical predictive variables for the Chilean and the Peale’s dolphins, all of which are representative of the places where the biopsy samples were taken, can be found in Appendixes 3 and 4, respectively.

3 - Results

3.1 - Case enrolment criteria

Only biopsy attempts that were successful (i.e. a tissue sample was collected) and in which sufficient visual and video data were obtained (Chilean dolphin = 32, Peale's dolphin = 75) were included in this evaluation (Graphic 1).

Graphic 1 - Absolute frequency of biopsies collected from each species: Ce - Chilean dolphin; La - Peale's dolphin.



3.2 - Survey effort and reported data

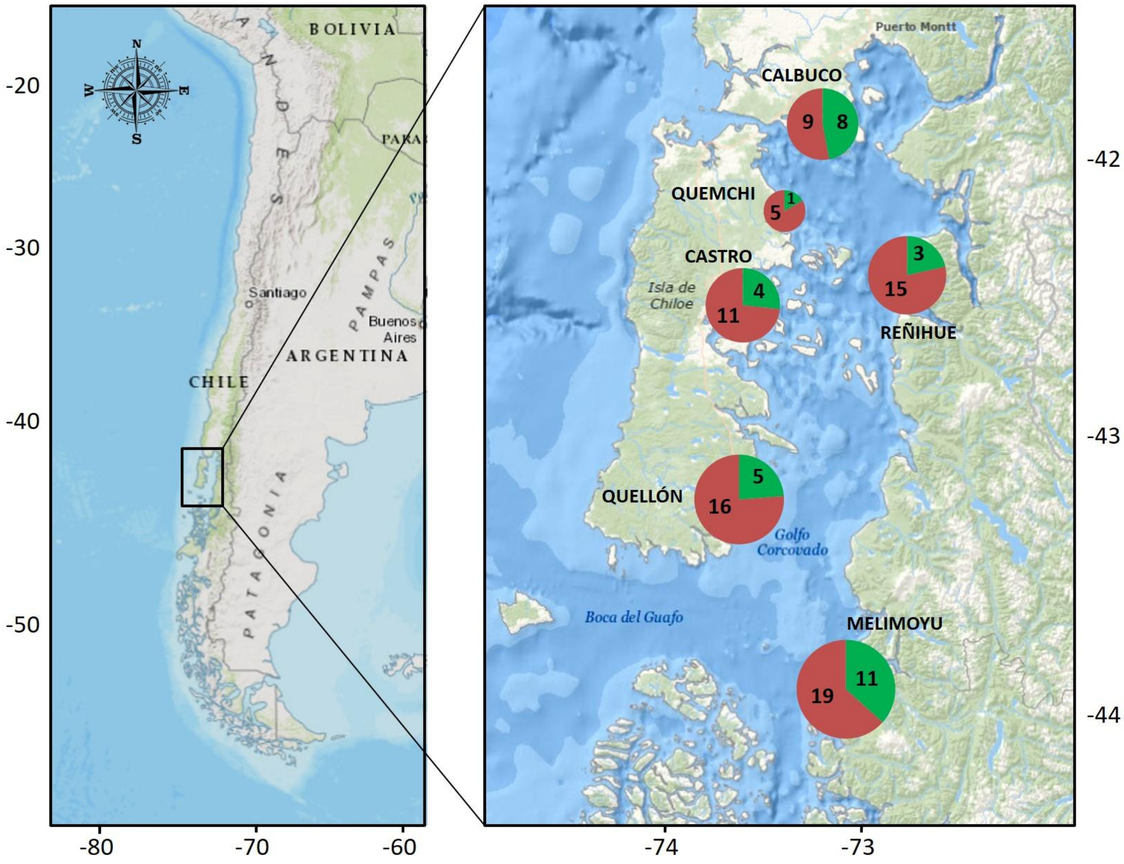
A total of 882h 55min were spent at sea, resulting in 79h18min of biopsy encounter time, over 106 days of study. During this time a total of 297 Chilean (Ce) and 245 Peale's (La) dolphin groups were sighted in the study area.

Although 297 groups of Chilean dolphins were sighted, and 46 animals were sampled, only 32 individual responses were video recorded and from these only 26 group reactions could be properly assessed. Regarding the Peale's dolphin we biopsied 90 animals, from which we were able to register 75 individual responses and 63 group reactions (Table 7; Figure 25).

Table 7 - Absolute frequency of behavioural responses recorded of both dolphin species (Chilean and Peale's dolphin) per area and according to reaction type: IR – individual reaction; GR – group reaction.

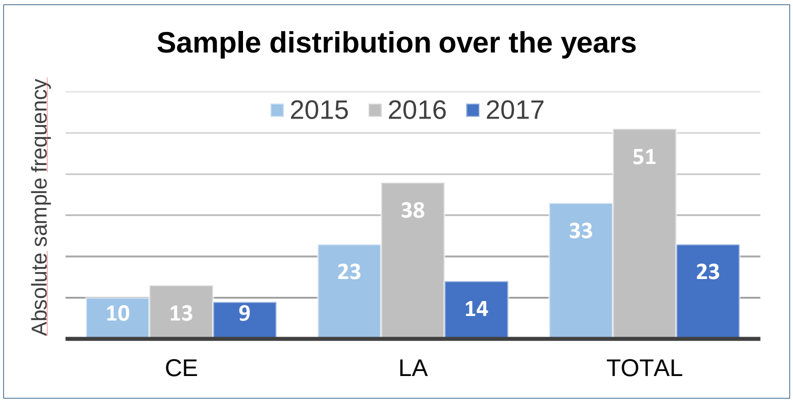
	Chilean dolphin		Peale's dolphin	
Location	IR	GR	IR	GR
Calbuco	4	4	11	10
Castro	8	5	9	8
Melimoyu	5	3	16	13
Quemchi	1	1	5	5
Quellón	11	10	19	16
Reñihue	3	3	15	11
Total	32	26	75	63

Figure 25 - Map of sampling locations in northern Chilean Patagonia. Number of biopsy samples for Chilean dolphins and Peale's dolphins is shown in green and red, respectively. The size of the chart is proportional to the total number of dolphins sampled in each population.



The distribution, by year, consisted in 33 animals sampled in 2015, 51 animals sampled in 2016 and 23 animals in 2017 (Graphic 2). According to season we sampled 32 animals in the summer (9 Ce and 23 La) and 75 during fall (23 Ce and 52 La).

Graphic 2 - Absolute sampling frequency over the years and by species. CE - Chilean dolphin; LA - Peale’s dolphin.



For both species, before each sampling attempt, the group of dolphins was monitored for approximately 21 minutes ($\overline{x}_{Ce}$ = 21.46 min, SD_{Ce} = 15.72, n_{Ce} = 26; $\overline{x}_{La}$= 21.71 min, SD_{La} = 19.64, n_{La} = 66). During this period, we noted their pre-biopsy behaviour (Appendixes

2 & 3), took the identification photos and decided which animals were suited for the technique (i.e. had notorious marks or scars on their body, mainly on their dorsal fin).

The contact period included the photo-identification efforts, representing the duration of all disturbance involved in the biopsy procedures (Best et al., 2005). Its mean was of 38 minutes and 8 seconds ($\bar{x}_{Ce}$ = 37min17sec, n_{Ce} = 25; $\bar{x}_{La}$ = 38min 58sec, n_{La} = 67) and ranged from 4 to 128 minutes (Table 8).

Table 8 **-** Descriptive analysis of the numeric variables recorded for both species: a - Chilean dolphin, b - Peale's dolphin. In order, the categories analysed were: prebiopsy monitoring period; duration of the whole encounter; size of group where sampling occurred; distance to shore; ocean depth; water visibility; Beaufort wind force scale; and distance to human activities/structures (e.g. fish and mussel farms, transport and fishing boats). The last 4 variables were taken at the exact location of the biopsy sampling.

a.

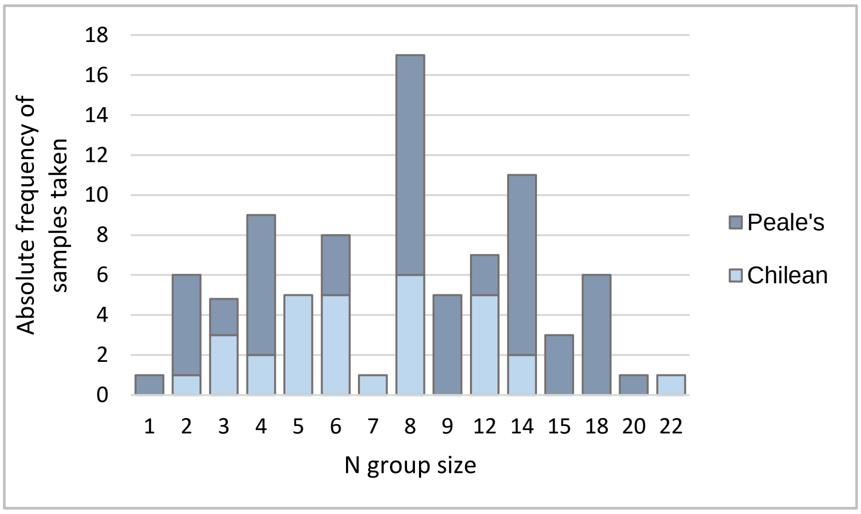
Descriptive Analysis	**CHILEAN DOLPHIN**							
Categories	**Prebiopsy (min)**	**Duration (min)**	**Group Size**	**Shore (m)**	**Depth (m)**	**Visibility (m)**	**Bft**	**Human activities (m)**
N	26	25	31	28	29	29	31	18
Min	1.00	12.00	2.00	24.00	1.60	1.50	0.00	100.00
Max	56.00	71.00	22.00	387.00	40.90	11.50	3.00	900.00
Mean	21.46	37.28	7.71	162.61	10.85	3.89	0.81	440.72
Standard deviation	15.72	16.19	4.30	92.71	10.73	2.20	0.65	298.44

b.

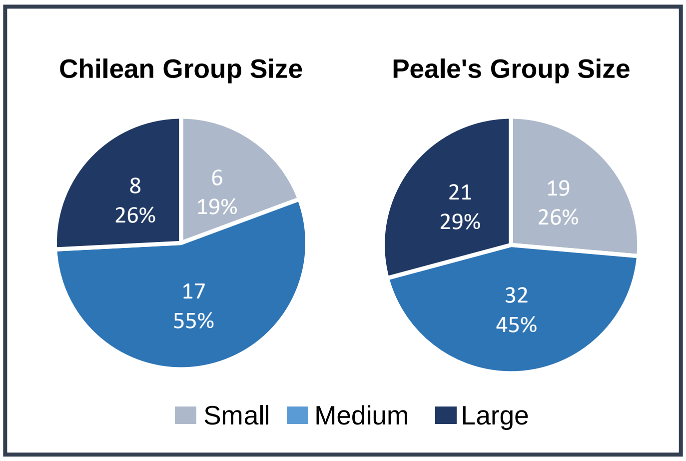
Descriptive Analysis	**PEALE'S DOLPHIN**							
Categories	**Prebiopsy (min)**	**Duration (min)**	**Group Size**	**Shore (m)**	**Depth (m)**	**Visibility (m)**	**Bft**	**Human activities (m)**
N	66	67	72	63	58	59	72	31
Min	0.00	4.00	1.00	60.00	1.90	1.90	0.00	44.00
Max	82.00	128.00	20.00	3000.00	72.90	9.00	3.00	1137.00
Mean	21.71	38.97	8.36	488.11	13.80	4.91	1.17	411.77
Standard deviation	19.64	26.15	5.09	771.75	15.81	1.93	0.71	359.22

Considered only the groups from which biopsies were taken, the Chilean dolphin's group size ranged from 2 to 22 individuals ($\bar{x}_{Ce}$ = 7.71, SD = 4.30, n = 31), similar to the Peale's dolphin whose range varied from 1 to 20 dolphins ($\bar{x}_{La}$ = 8.36, SD = 5.09, n = 72) (Graphic 3). Although our group scope for the Chilean dolphin was slightly larger than described in the literature (Heinrich, 2006), the Peale's dolphin group amplitude was as characterized, with the most frequently observed number of animals being of 8 per group. Allegedly Chilean dolphins are usually seen in smaller groups than Peale's dolphins, however in our study we found no statistical differences between species and group size (Fisher's exact test, p = 0.75) (Graphic 4). We think this was because small groups of Chilean dolphins showed to be very hard if not impossible to be sampled. Given bigger groups of dolphins were more accessible we ended up having a biased group size of sampled animals, by excluding several smaller groups from the analysis.

Graphic 3 - Absolute frequencies of samples taken per group size and per species.

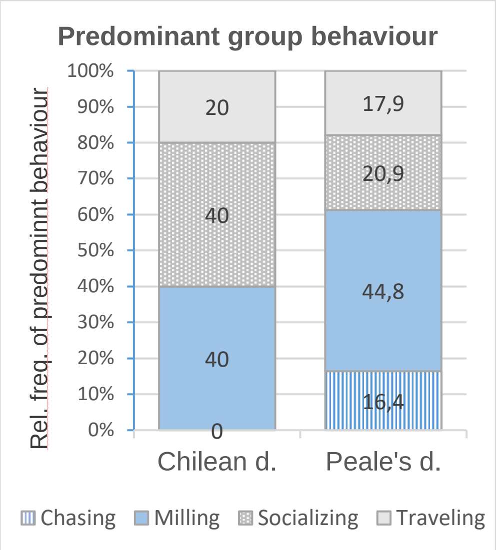


Graphic 4 - Absolute and relative frequencies of samples taken per category of group size and per species. Small includes 1-4 animals. Medium includes 5 -9 animals. Large are groups above 10 animals.

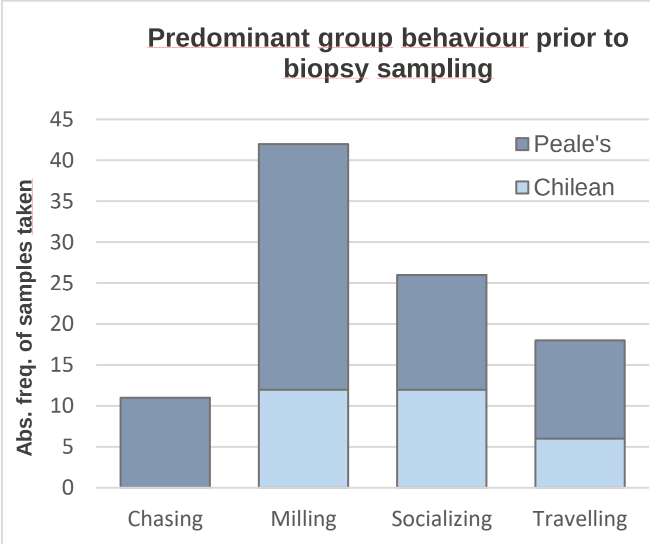


The predominant group behaviour differed between species (Fisher exact test, p = 0.035). Only Peale's dolphins were seen chasing and they seemed to spend less time socialising when compared to its sympatric species (Graphics 5 & 6). Overall, the most frequently seen behaviour in either species was milling (CE=40%; LA=45%), followed by socializing (CE=40%; LA=21%) and travelling (CE=20%; LA=18%).

Graphic 5 - Relative frequencies (%) of predominant group behaviour according to species.



Graphic 6 - Absolute frequencies of predominant group behaviour according to species.



The distance to shore was expressively different between species. The Chilean dolphins had a mean distance of 162.61 meters from the coast (SD_{CE} = 92.71, n = 28) whereas Peale's dolphin groups were mainly spotted 488.11 meters off the coast (SD_{LA} = 771.75, n = 63). Even though it wasn't significant (Kruskal-Wallis, χ^2 = 2.72, df = 1, p = 0.099) it might have been simply due to our small sample data.

The visibility of the water was in average no more than 4,4 meters ($\bar{x}_{Ce}$ = 3.89m, SD_{Ce} = 2.20, n_{Ce} = 29; $\bar{x}_{La}$= 4.91m, SD_{La} = 1.93, n_{La} = 59), on the other hand, the depth of the ocean floor was of approximately 12 meters ($\bar{x}_{Ce}$ = 10.85m, SD_{Ce} = 10.73, n_{Ce} = 29; $\bar{x}_{La}$ = 13.80m, SD_{La} = 15.81, n_{La} = 58). This explains the difficulty found in gathering good quality video where the visibility would allow a proper behavioural assessment. As referred in the literature, both of these species have a coastal distribution and that is particularly noticeable on graphic 7, showing the majority of the animals were found in depths less than 20m.

Regarding human interaction, we recorded every human construction or activity that would be in the near radius of the group being sampled (Appendix 5). The Chilean dolphins sampled were seen in average about 418.3 meters away from these structures (SD_{Ce} = 336.3, n_{Ce} = 16) while the Peale's dolphins' groups were seen slightly closer, at 411.8 m (SD_{La} = 349.5, n_{La} = 30). It is evident that animals aren't static and that they move freely within their distribution, regardless it is noteworthy that the areas considered "disturbed" presented a higher number of verified human activities compared to the "natural" sites (Table 9), a record on the substantial overlap with these species' habitat.

Graphic 7 - Histogram of the absolute frequency of biopsy samples collected per depth level and according to the species.

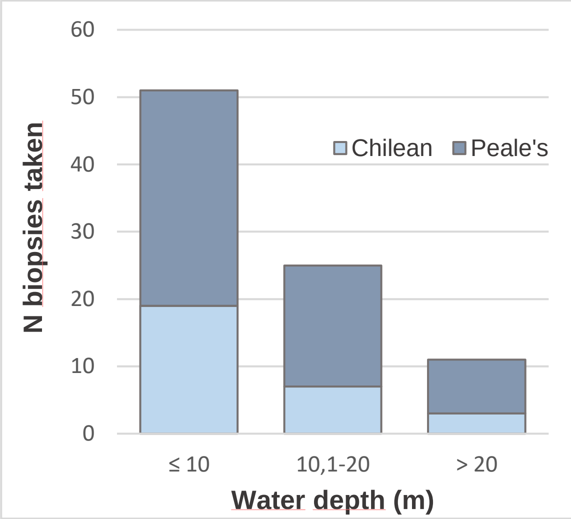


Table 9 - Absolute and relative frequencies of sampled dolphins in general, and sampled dolphins near human activities in particular, per type of area studied (disturbed versus natural).

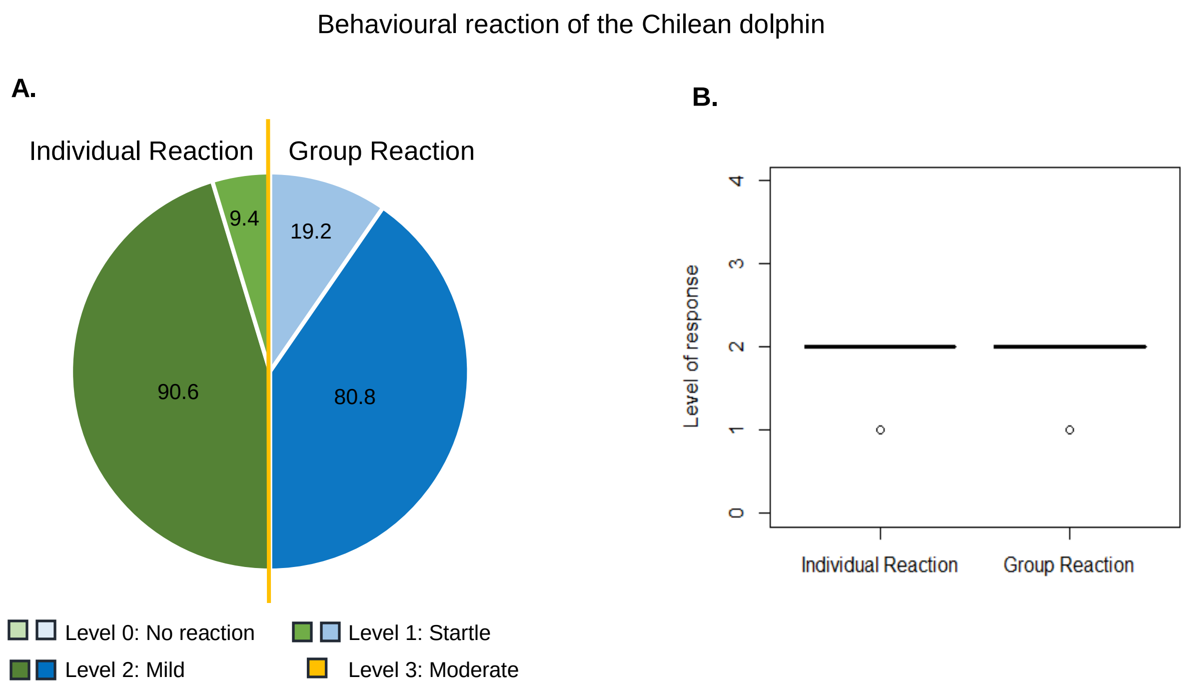
		Chilean dolphin				**Peale's dolphin**			
Area		**n**	**%**	**Human activities**	%	**n**	**%**	**Human activities**	%
Disturbed	Calbuco, Castro, Quellón, Quemchi	18	56.3	10	62.5	41	54.7	22	48.9
Natural	Melimoyu, Reñihue	14	43.7	6	37.5	34	45.3	8	23.5
Total		32	100	16	50	75	100	30	40

In the disturbed areas (Calbuco, Castro, Quellón and Quemchi), eleven out of the twenty-two Peale's dolphin sampled were seen close to clam-harvesting boats ($\overline{x}$ = 553.3m). Performing 50% of the dolphins sampled, these reports support our claim that these are high marine traffic areas. The remaining animals were seen close to mussel farms ($\overline{x}$ = 158.2m), another main economic activity in the region. Furthermore, and as previously mentioned, Chilean dolphins are usually seen closer to coast in sheltered bays, where the mussel and salmon farms are also located, overlapping with this species habitat. Eight out of twenty Chilean dolphins were seen close to, and even interacting with, mussel or salmon farms (40%, $\overline{x}$ = 498m).

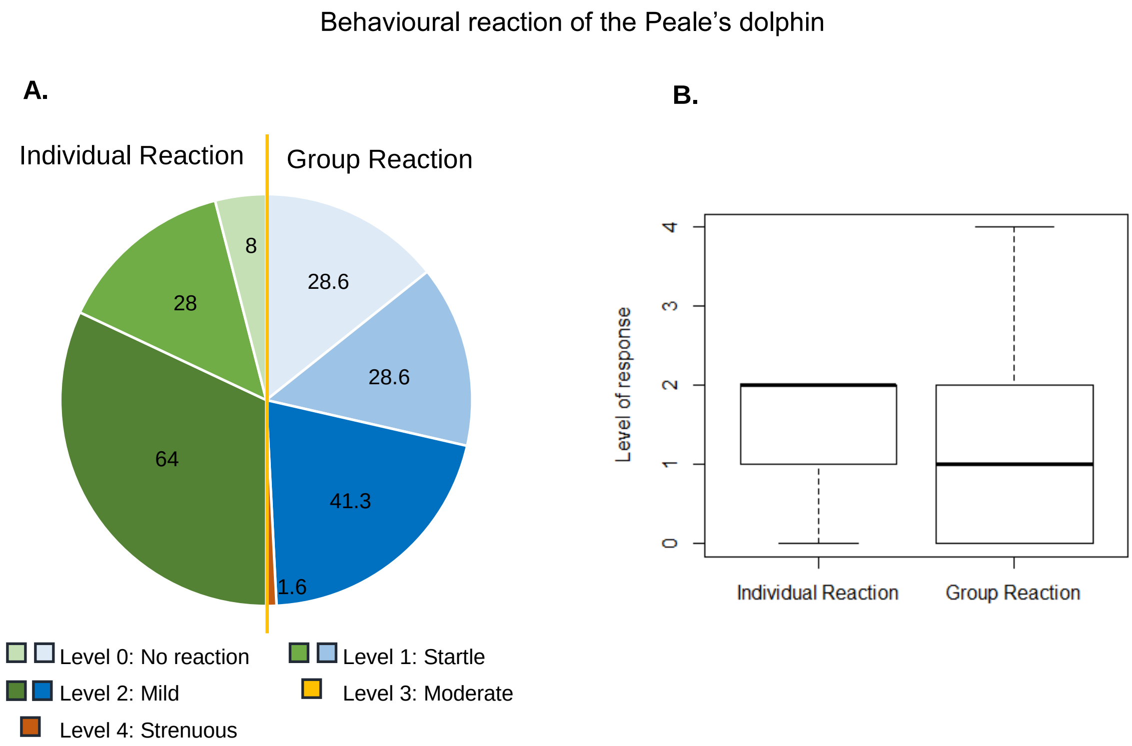
3.3 - Behavioural reactions to biopsy sampling

The Chilean dolphin exhibited the same individual reaction, level 2, in 90.6% of the sampling events, and level 1 in the remaining 9.4% (Graphic 8), while the Peale's dolphin had a higher diversity in responses being the category 2 the most frequently seen (64.0%), followed by category 1 (28.0%), and finally category 0 (8.0%) which entails no response to the procedure (Graphic 9). There were no strenuous reactions recorded from any of the individuals targeted as in Hooker et al. (2001). Concerning the group reactions, the Chilean dolphin presented high correlation with the individual reactions, displaying category 2 responses in 80.8% of the biopsy attempts and category 1 reactions in the remaining 19.2%. The Peale's dolphin group reaction was, as for the individual reactions, more heterogeneous, being category 2 the most frequently recorded reaction (41.3%), followed by 0 and 1 (both at 28.6%) and one strenuous level 4 reaction (1.6%) that consisted of two leaps performed by the dolphin that was right next to the targeted animal.

Graphic 8 - Frequency of immediate behavioural reactions of the Chilean dolphin. A) Relative frequencies (%) of individual and group immediate reactions recorded from Chilean dolphins to our biopsy pole system. B) Box plot of the individual and group immediate reactions of the Chilean dolphin to the biopsy pole system.

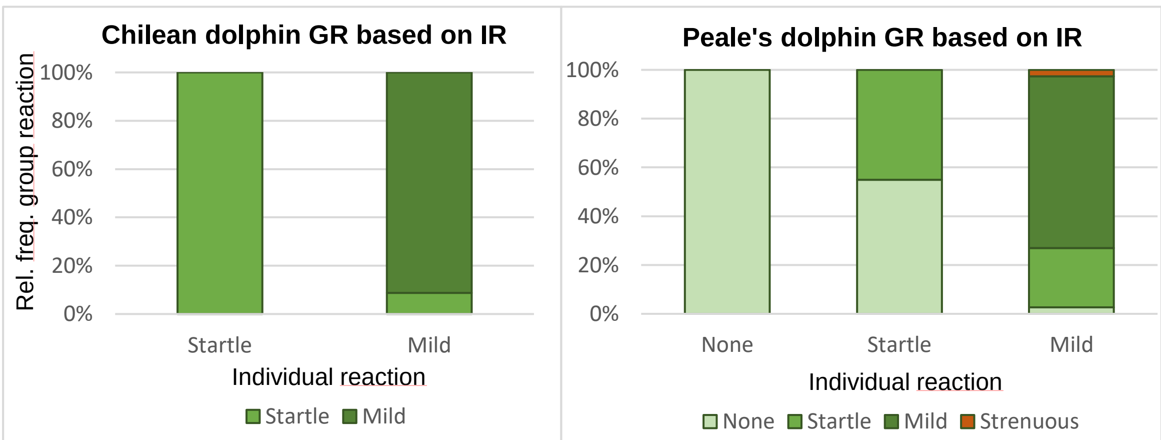


Graphic 9 - Frequency of immediate behavioural reactions of the Peale's dolphin. A) Relative frequencies (%) of individual and group immediate reactions recorded from Peale's dolphins to our biopsy pole system. B) Box plot of the individual and group immediate reactions of the Peale's dolphin to the biopsy pole system.



As soon as the biopsied dolphin responded to our technique the group followed. In the case of the Chilean dolphin there were no group reactions of higher intensity that the one shown by the targeted animal. Regarding the Peale's dolphin whenever the targeted dolphins reacted at a mild intensity (level 2) the group reacted mainly in the same way or at an inferior level, except for one recorded strenuous reaction (level 4).

Graphic 10 - Relative frequencies for group reaction (GR) dependent upon individual reaction (IR). Level 0 = "none"; level 1 = "startle"; level 2 = "mild"; level 4 = "strenuous".

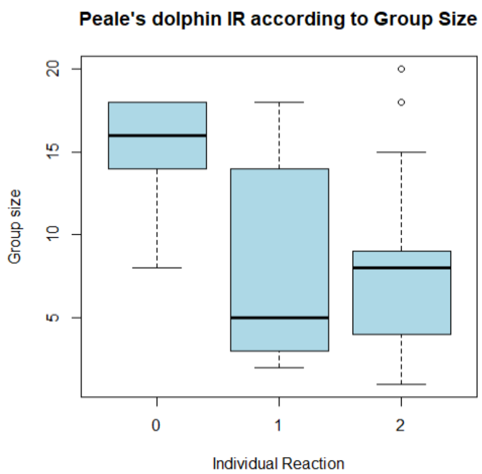


The group response seems to be intimately dependent upon the individual response (Fisher's exact test, $p < 0.001$). Attending to Graphic 10, if the targeted animal does not react, the group does not either. This may justify why we always recorded a group response for the Chilean dolphin, since all these individuals reacted.

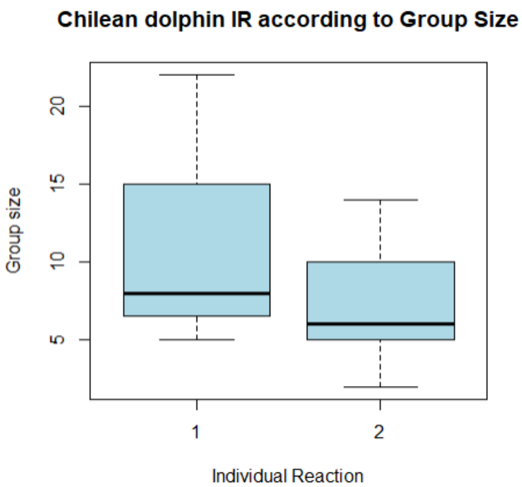
We noted there was a significant variation between species regarding the individual reaction and the group reaction (Fisher's exact test, $p < 0.05$ and $p < 0.001$ respectively). The Chilean dolphins always reacted to our disturbance. They also revealed to be very consistent in the intensity of their response to the technique, both at individual and group levels, showing little plasticity. Furthermore, the Chilean dolphins are inconspicuous and of elusive behaviour, making it harder to follow their movements and acquiring a biopsy sample. This shyness may rend them to be more sensitive to our disturbance than the Peale's dolphin and thus possibly justifying the 100% rate of reaction to the biopsy sampling technique.

The Peale's dolphins' group response was significantly different between group sizes (Kruskal-Wallis, $\chi^2 = 9.220$, df = 2, p = 0.010) (Graphic 11) which wasn't the case of the Chilean dolphin (Graphic 12). After performing the appropriate post-hoc test, we confirmed the differences between level 0 and both levels 1 and 2 (Dunn test, respectively, p = 0.001 & p =0.003). All situations where dolphins didn't react occurred in groups bigger than 8 individuals, and mostly large groups (>10 animals). Regardless of the group size (S, M, L), all individual responses presented by the Peale's dolphin had the same pattern: most frequent reaction was level 2 (58.8%, 70% and 42.9% respectively), followed by level 1 (41.2%, 26.7% and 35.7%) and finally level 0. However, it is noticeable that the bigger the group the higher the percentage of none responsive animals (Graphic 13). In regard to the Chilean dolphin, even though they always reacted to the technique, it is also apparent that larger groups react less intensely (Graphics 13 & 14). It may be that in such groups the animals distract one another more and hence the individual targeted fail to notice our biopsy.

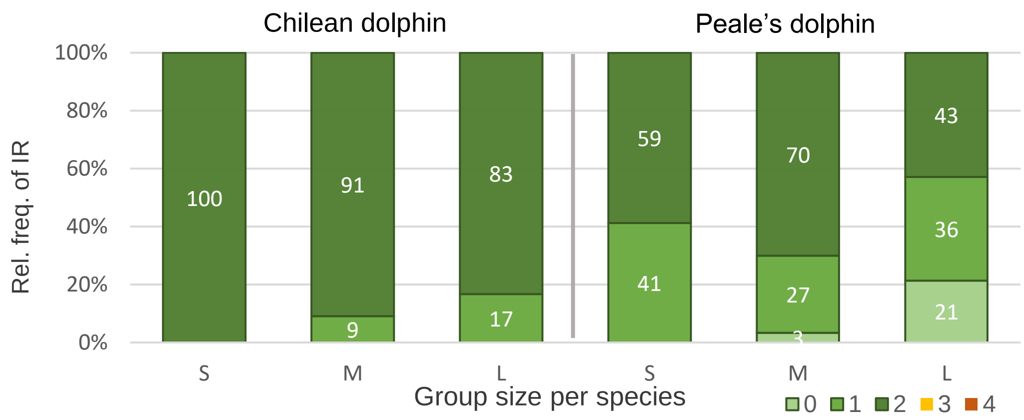
Graphic 11 - Box and whiskers plot of Peale's dolphins' individual reaction according to group size.



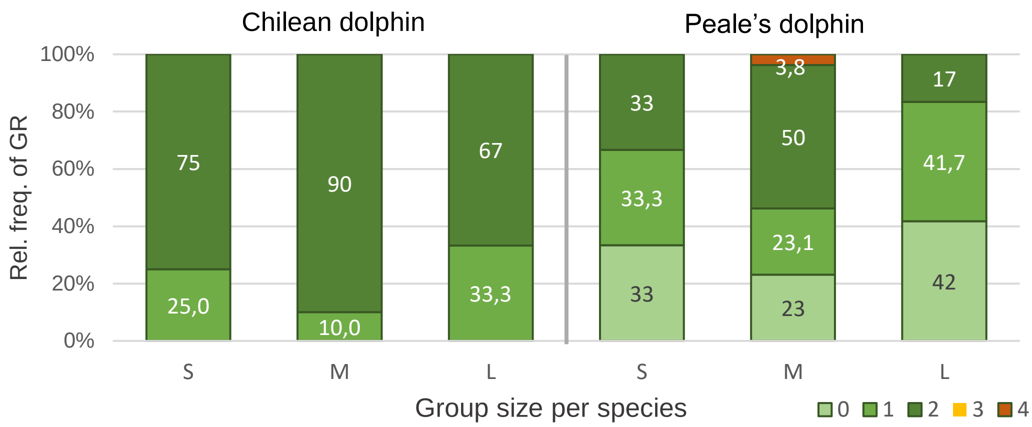
Graphic 12 - Box and whiskers plot of Chilean dolphins' individual reaction according to group size.



Graphic 13 - Relative frequencies (%) of intensity of individual response (IR) to the biopsy technique based on group size. S - Small (1 to 4 individuals); M - Medium (5 to 9 individuals), L - Large (equal or more than 10 individuals).



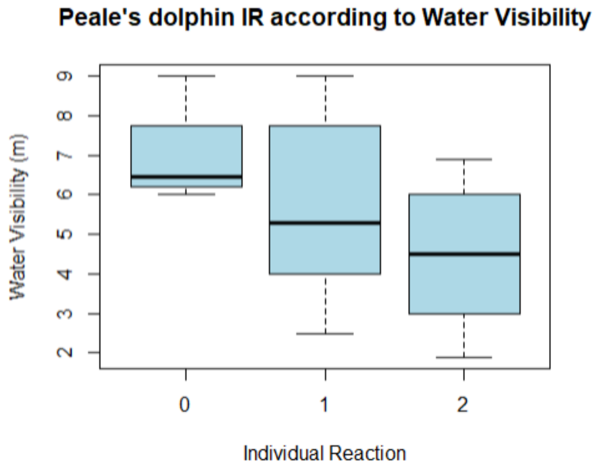
Graphic 14 - Relative frequencies (%) of the intensity of groups' response based on group size. S - Small (1 to 4 individuals); M - Medium (5 to 9 individuals), L - Large (equal or more than 10 individuals).



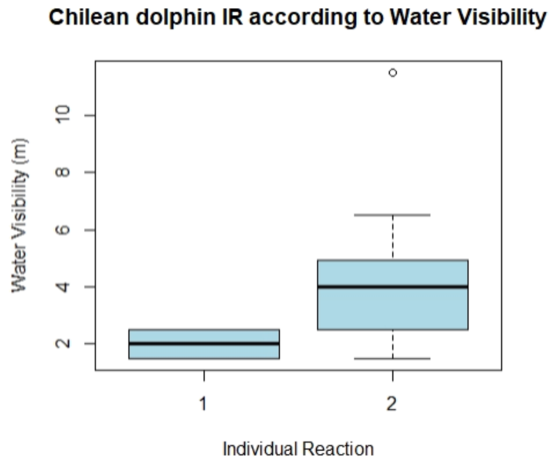
When considering the intensity of reaction of the dolphin next to the targeted animal (group reaction) despite the lack of statistical difference it appears to exist a tendency for both species medium sized groups to react more intensely than the other groups (Graphic 14). Curiously, the only strenuous response recorded was from a medium sized Peale's dolphin group. Furthermore, it seems that animals that do not react (category 0) seem to occur more frequently in large groups ($\bar{x}$ = 14), while those who react more vigorously are found in smaller groups ($\bar{x}$ = 6). Overall there is a tendency for larger groups to react less intensively at both individual and group levels.

The visibility of the water was found to be significantly higher when the lack of individual reactions from the Peale's dolphins were recorded (Kruskal-Wallis, χ^2 = 9.532, df = 2, p = 0.009) with a mean rank of 6.98 m for "No reaction" (level 0), 5.72 m for "Startle reaction" (level 1), and 4.36 m for "Mild reaction" (level 2) (Graphic 15). After performing a post-hoc test (Dunn test) we were able to confirm a difference between level 2 and both levels 1 and 0 (p = 0.005). Regarding the Chilean dolphin, it appears that mild reactions were mainly seen at around the same visibility range as the Peale's dolphin (x̅=4.03m), however, startle reactions were shown at a really short visibility range (x̅=2m) (Graphic 16).

Graphic 15 - Box and Whiskers Plot relating the Peale's dolphin individual reaction with the water visibility (in meters) of where the biopsy procedure

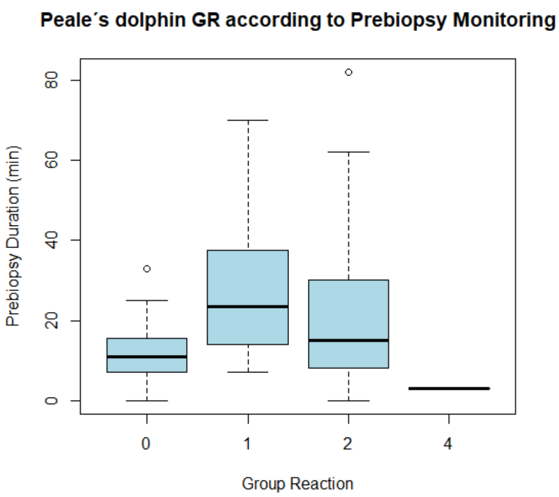


Graphic 16 - Box and Whiskers Plot relating the Chilean dolphin individual reaction with visibility of the water (in meters) where the

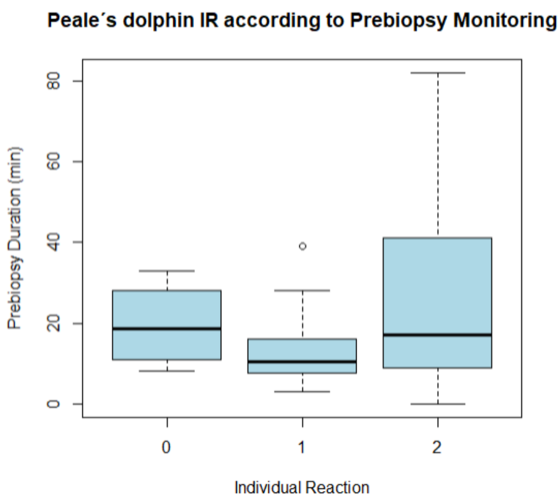


We found no dependency between the intensity of the individual reaction and the duration of the prebiopsy monitoring for any of the species, yet the group reaction was quite different for the Peale's dolphin (Graphic 17), particularly between levels 0 and 1 (Dunn test, $p = 0.017$). It is noteworthy that all level 0 individual reactions happened when the monitoring period prebiopsy was inferior to 35min and group reactions when it was under 25min, suggesting that maybe the best time-frame for pre-monitoring would be under 25 minutes (Graphic 18).

Graphic 17 - Box and Whiskers Plot relating Peale's dolphin group reaction (GR) with prebiopsy monitoring period.



Graphic 18 - Box and Whiskers Plot relating the Peale's dolphin individual reaction (IR) with prebiopsy monitoring period.

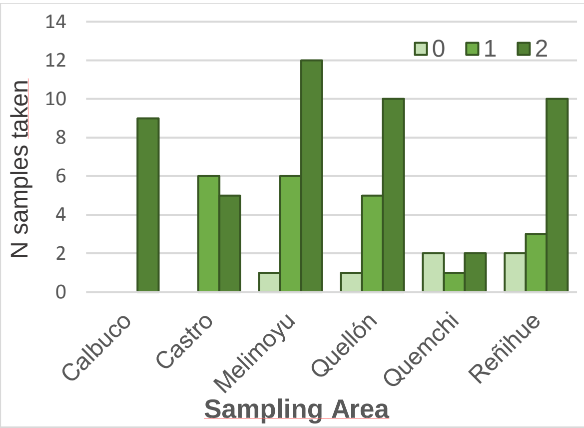


The duration of the encounters with the Peale's dolphins, revealed the longest encounter durations (>1h) had a bigger proportion in mild reactions (level 2) on both individual and group levels. This tendency suggests the amount of time spent with the groups may elicit stronger reactions. Overall, we would advise researchers to avoid following groups for periods over 40minutes.

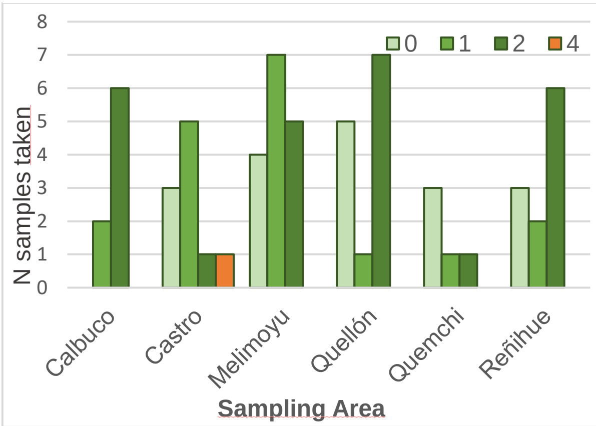
Lastly, we analysed if being biopsied near human structures or activities could influence intensity of reaction to the biopsy technique. We found no significant difference in both individual (KW, $\chi^2 = 1.859$, df = 2, p-value = 0.395) and group responses (KW, $\chi^2 = 2.494$, df = 2, p = 0.287).

It seems that throughout the different sampling areas, either type of reaction (individual and group) is dependent upon its particular ecological context (Graphics 19 & 20). Nonetheless, it is important to highlight that the pattern we found on group reactions being less intense than individual ones, is seen throughout the whole area range (except for the one strenuous reaction recorded in Castro).

Graphic 19 - Peale's dolphin individual reactions according to location where the sampling occurred.

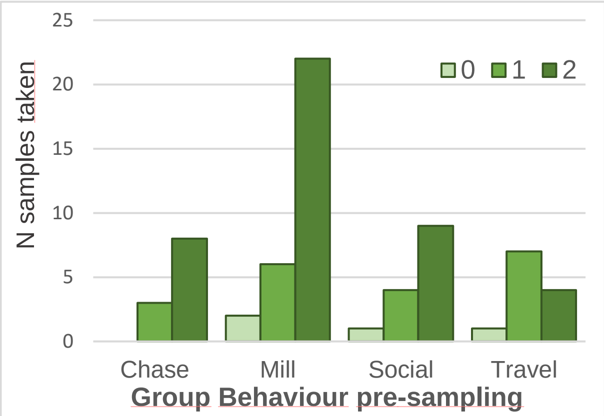


Graphic 20 - Peale's dolphin group reactions according to location where the sampling occurred.

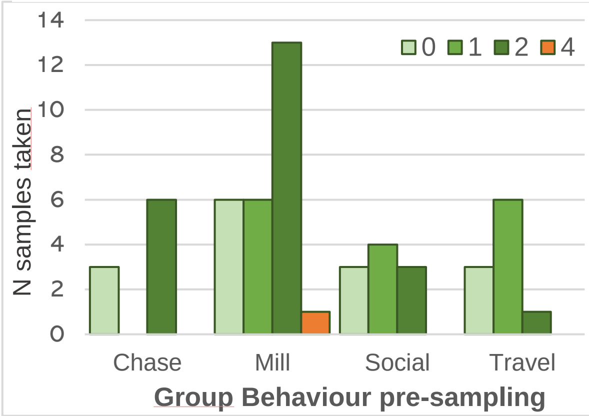


When looking into the Peale's dolphin group behaviour registered before the sampling, we can find the all types of reaction throughout the different states of behaviour (Graphics 21 & 22). However, it seems it is more likely to obtain reactions of lighter intensity in groups which are travelling. Additionally, most biopsy samplings were collected while the animals were milling, although it resulted in worse behavioural responses, mostly level 2 reactions and the one level 4 reaction. On the other hand, in general, the response magnitude appeared to decrease at the group level, particularly when animals were found socializing before the procedure.

Graphic 21 - Peale's dolphin individual reaction according to pre-sampling group behaviour displayed (by order: chasing; milling; socializing and travelling).

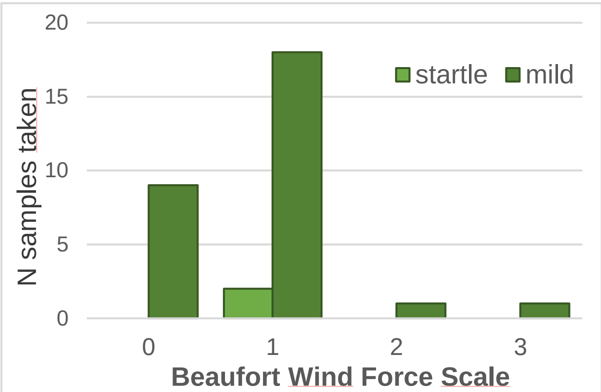


Graphic 22 - Peale's dolphin group reaction according to pre-sampling group behaviour displayed. (by order: chasing; milling; socializing and travelling).

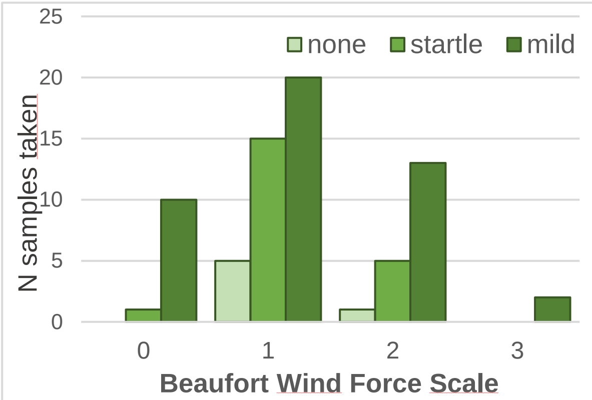


The majority of the sampling occurred on a Beaufort state 0 and 1 (Graphics 23 & 24). Even though the number of Chilean dolphins sampled was really low when compared to the number of Peale's dolphins it is interesting that the proportion of samples obtained from the first on a Beaufort state 0 was higher than from the latter. This might be because we usually encountered the Chilean dolphins closer to shore, where sheltered bays can prevent the wind and thus sea state to worsen.

Graphic 23 - Chilean dolphin individual reaction according to Beaufort scale at the moment of sampling.



Graphic 24 - Peale's dolphin individual reaction according to Beaufort scale at the moment of sampling.

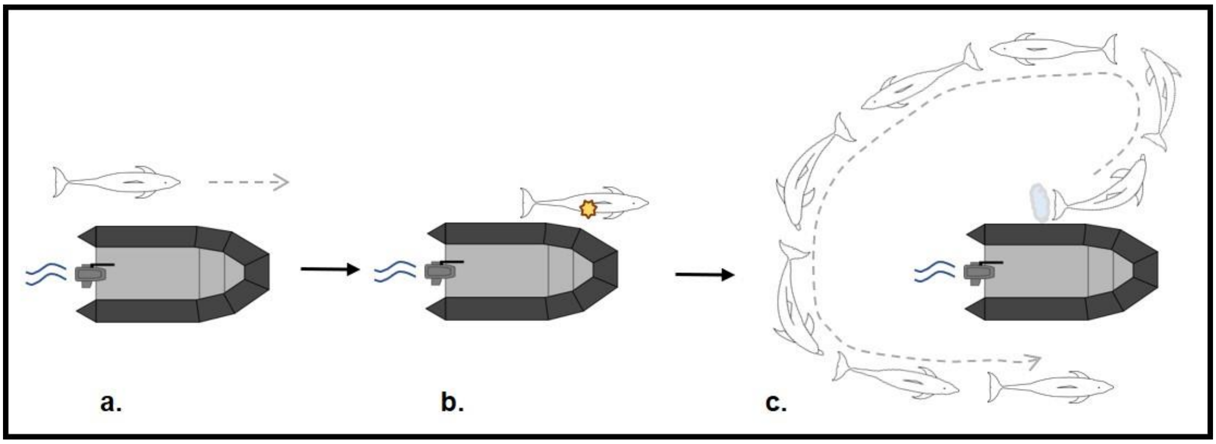


Description of the surface short-term behaviour of the Peale's dolphin to the biopsy pole system:

After the group of dolphins is sighted and approached, the target animal gets selected. The boat starts to travel parallel to the group's movement direction and approximately at the same speed, which usually encouraged the dolphins to bow-ride (Figure 26a.). When the target animal would find itself at an optimal angle, close to the bow and with no affiliate between it and the researcher, a biopsy sample would be taken with the pole on the side of the animal that was facing the vessel (e.g. sample acquired on the right side of the dolphin on Figure 26b). Immediately after the biopsy was attempted the animals usually reacted in one out of two ways:

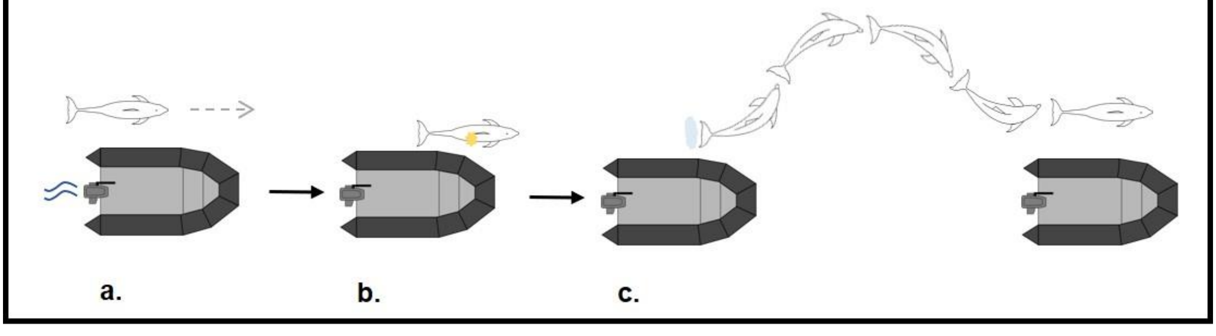
Boomerang response (Figure 26): The sampled animal accelerates away from the impact site, turning 180º towards the stern and crosses behind the vessel to the other side of it. Shortly after it returns to its bow-riding position but in the contralateral side of the vessel.

Figure 26 - Sequential depiction the Peale's dolphin's Boomerang response: (a) approach of the dolphin to the research boat, (b) moment when the biopsy sample occurs, (c) the animal moves away from the stimuli area but quickly returns to the vicinity of the boat.



Flinch response (Figure 27): The sampled animal gets startled by the stimuli but never leaves the vicinity of the boat. It simply swims away and shortly returns to bow-ride closely, always swimming the same direction as the research vessel.

Figure 27 - Sequential depiction the Peale's dolphin Flinch response: (a) approach of the dolphin to the research boat, (b) moment when the biopsy sample occurs, (c) the animal gets startled but quickly returns to his previous position.

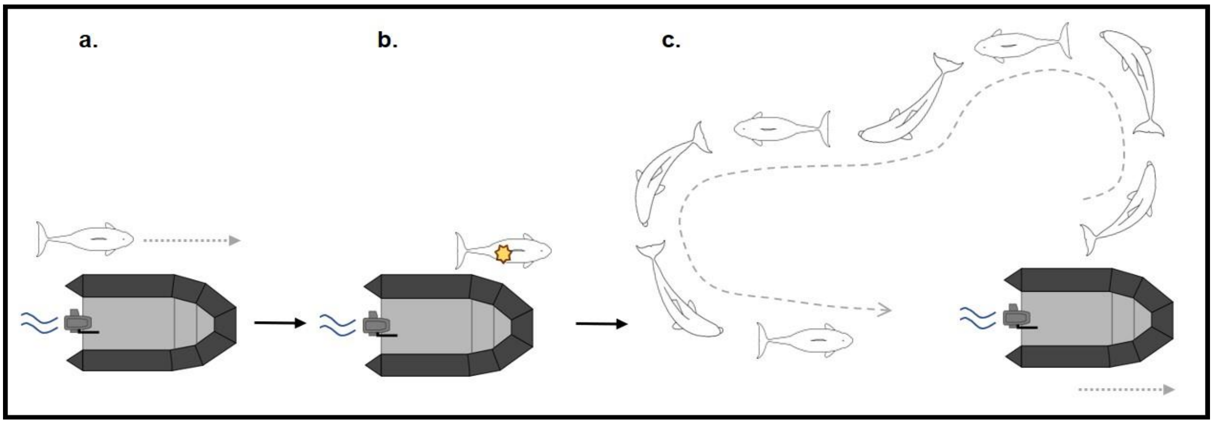


Description of the surface short-term behaviour of the Chilean dolphin to the biopsy pole system:

After the group of dolphins is sighted and approached, the target animal gets selected. The boat starts to travel parallel to the group's movement direction and approximately at the same speed, motivating the animals to bow-ride (Figure 28a.). When the target animal would find itself at an optimal angle, close to the bow and with no affiliate between it and the researcher, a biopsy sample would be taken with the pole on the side of the animal that was facing the vessel (e.g. sample acquired on the right side of the dolphin on Figure 28b.). All dolphins were sampled under the water surface. Immediately after the biopsy was attempted the animals usually reacted in on out of two ways:

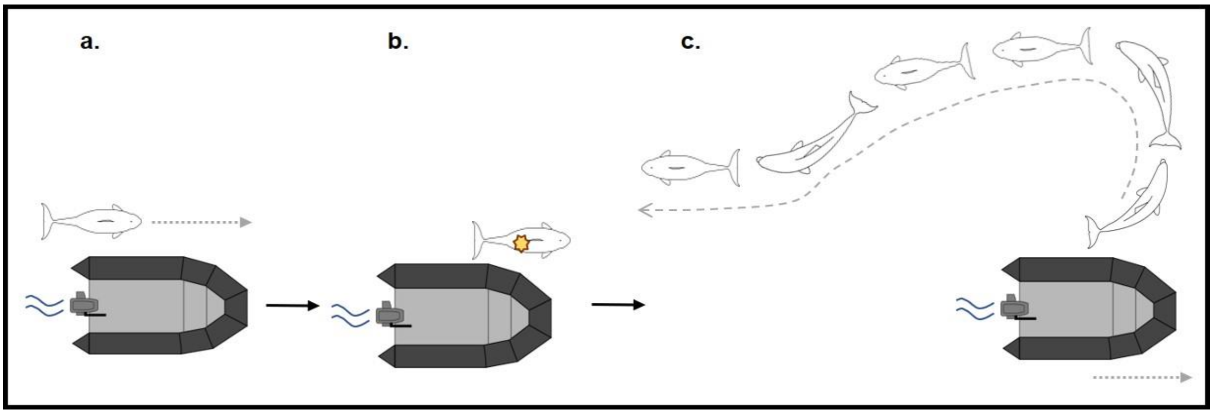
Recoil response (Figure 28): The sampled animal accelerates away from the vessel, turning 180º towards the stern. It swiftly returns to the boat's vicinity, adopting the same direction and speed, although following it from behind.

Figure 28 - Sequential depiction of the Chilean dolphin's Recoil response: (a) approach of the dolphin to the research boat, (b) moment when the biopsy sample occurs, (c) the animal moves away from the stimuli area but quickly returns to the vicinity of the boat.



Stern Flight response (Figure 29): The sampled animal accelerates away from the stimuli, turning 180º towards the stern, and returns to its initial geographical position from before the disturbance started, which was a few meters behind.

Figure 29 - Sequential depiction of the Chilean dolphin's Stern flight response: (a) approach of the dolphin to the research boat, (b) moment when the biopsy sample occurs, (c) the animal accelerates, moving away from the stimuli.



4 - Discussion of results

4.1 - General findings

Sampled Chilean dolphins occurred mainly within 200 meters from shore, and in shallow water (≤10m), which confirms what has been described in the literature (Heinrich, 2006; Viddi, 2015). Sampled Peale's dolphins occurred in a similar range of depth (approximately 14m), however its distance to shore was expressively different, mainly within 500m but with sights as far as 3000m from the shore. These findings conform to the broad distribution and movement range stated by different authors (Heinrich, 2006; Hammond et al., 2008) that the Peale's dolphin has a higher flexibly in habitat use when compared to the Chilean dolphin.

Attending to the fact that both species are truly coastal, requiring researchers to perform their field methodologies close to shore, thus being more visible, it is important to be careful and, if possible, avoid the presence of the public eye (passive observers). This because the general public may misunderstand these procedures, which are carefully designed by experts, and attempt to replicate it themselves, consequently distressing, if not hurting, the dolphins. Another reason to be mindful when working near the shore, is that in this region, throughout its coast line, it is common the use of intertidal fishing nets, which may pose serious risk of injuries, or even death, to the animals if they get close to them. So, before starting all the practical work, it is really important to scan the area where the encounters could take place. Lastly, while working at depths as shallow as 1.6 m, it is crucial to be aware of the topography of the bottom, in order to avoid collisions with rocks, entanglement in seaweeds or even accidentally hurting the animals.

For every sighting, and prior to sampling, we recorded the predominant behaviour that the group would be engaged. For the Chilean dolphin, the most frequently seen was both milling and socializing followed by travelling. In Heinrich's observations (2006) the prevailing behaviour was milling and the least recorded was socializing, as for Viddi (2015) his most recorded behavioural state was travelling, followed by milling. Both authors remarked on how little time this species seemed to invest in social activities, and yet most of our sampling was done on groups doing exactly so. Probably this was due to the fact that we would more easily biopsy sample groups that were either milling or socializing, thus increasing the frequency that those behaviours were recorded, in contrast to other states of behaviour whose groups weren't sampled and as such excluded from our records, thus biasing our overall results. Regarding the Peale's dolphin, the predominant group behaviour recorded was also milling, followed by socializing, travelling and lastly chasing. Which again contrasts with Heinrich (2006) and Viddi (2015) where travelling was the most frequently recorded behaviour, followed by milling and socializing, which we've already discussed that our sampled population cannot reflect the overall regional species population due to potential biases. Nonetheless, from our study's observations, the Peale's dolphin behaviour displayed did not seem to influence the biopsy sampling success as it appeared for the Chilean dolphin, but it did seem to influence the reaction to it, which will be discussed further ahead.

Both the Chilean and the Peale's dolphin show fluid social structures, so called fusion-fission societies, where groups temporarily associate to bridge different necessities (Prox, 2017). The first sppecies is usually seen in small aggregations, mostly ranging from 2 to 6 individuals (Goodall et al., 1988; Heinrich, 2006; Viddi, 2009), and yet in our study our mean group size was of 8 animals. This was probably a by-product of larger groups being easier to sample and thus distorting our estimates on most frequent group size. As for the second species, literature refers its groups are usually comprised of 4 individuals (Brownell et al., 1999, Heinrich, 2006; Viddi, 2009) and in our study we found mostly medium groups with 8 individuals. Again, this might be because larger groups are easier to sample but also because sometimes, we sampled more than one individual per group thus biasing our assessment. Nonetheless, the group ranges for both species were as described in the literature, between 2 and 22 for the Chilean dolphin and from 1 to 20 for the Peale's dolphin.

The sampled Chilean dolphins were seen at depths of particularly 10.9m but ranging from 1.6 m to 40.9 m, which generally conforms to what as been described (Heinrich, 2006). And the Peale's dolphins were mainly seen at sites 13.8 m deep, ranging from 1.9 m to 72.9m, following the same pattern detailed by Brownell et al. (1999) of a species which is mainly attracted to shallower coastal waters. Furthermore, the distance the sampled dolphins were from shore was of approximately 163 m for the Chilean dolphin and 488 m for the Peale's dolphin which is correspondent to coastal habitats, where both species are described to live in (Heinrich, 2006).

Most of the biopsies were acquired at a Beaufort scale of 0 and 1. This might be because these species do not exhibit an expressive surface activity and as such it might be easier to spot the dolphin groups at a Beaufort force smaller than 2. Additionally, it is interesting to note that all individual Chilean dolphins reacted strongly (level 2) at Beaufort 0, which could be because with a mirror-like sea it becomes easier to perceive the visual cues.

The wound caused by our biopsy punch was minimal and superficial, leading us to hypothesize it posed no harm to the animals' physical health, on a long-term perspective. Considering we used a smaller tip than Loizaga de Castro et al. (2013), which had 18mm in length until reaching the stop, it cannot penetrate deeper than the skin and blubber layers, thus reducing considerably the risk of severely injuring the animals, and the probability of becoming a vector for infections (Gorgone et al., 2008). Even though we couldn't analyse the healing duration due to time and mechanic constrictions, we can assume it would take about the same period as reported, approximately a month. Furthermore, a year later it remained only a white scar coverage where the animals had been biopsied the previous year (noticed in the 2016 field season regarding the dolphins sampled the year before), supporting the theory this procedure does not debilitate the animal's health or facilitate infectious diseases.

4.2 - Behavioural reactions to biopsy sampling

4.2.1 - Main findings

There are only four published studies which, like us, evaluated the behavioural response to a biopsy pole system (Marsili & Focardi 1996; Fossi et al. 2004; Bilgmann et al 2007; Loizaga de Castro et al., 2013). The results of these four experiments suggested the technique generally elicited startle to mild behavioural responses, regardless of the species. It is important to note that in all these studies the pole was either dropped on top of the animal's dorsal area or thrown lightly towards the animal. Therefore, this study is the first to use the power of an elastic band to impulse the pole towards the dolphin.

Our study's results support the cited literature (Noren & Mocklin, 2012) as the majority of the individual reactions were considered mild (90.6% for the Chilean dolphin and 64% for the Peale's dolphin) followed by a startle reaction (9.4% for the Chilean dolphin and 28% for the Peale's dolphin). As in previous studies (Weinrich et al. 1991; Jefferson & Hung 2008), the sampled animals generally accelerated and swam away from the vessel, but it was possible to approach them closely again within 2 to 5 min of sampling.

In regard to the group reactions, the same answer trend was verified. The Chilean dolphin displayed mild reactions (level 2) in 80.8% of the time and startle responses (level 1) in the remaining 19.2%. The Peale's dolphin had a more diverse range of group reaction, with "mild" being the most frequently recorded reaction (level 2, 41.27%), followed by "none" and "startle" (levels 0 and 1 respectively; both with 28.57%), and one strenuous reaction (level 4, 1.59%) that consisted of two energetic leaps while swimming away from the boat. In general, there was a decrease in intensity from individual to group reactions.

The group response was intimately dependent upon the individual response. Once the targeted dolphin reacted to the biopsy punch, the group followed. Except for one situation, there were never group reactions of higher intensity that the one shown by the targeted animal. In the Peale's dolphin case, this dependency was even more evident since whenever the sampled animal failed to respond (level 0), the group did not either. It could be possible that, from a chronological and biological point of view, the animal being sampled would be the first one to react to it, by perceiving the procedure on itself through its somatosensory system, and thenceforth the responsible for the transmission of some kind of acoustic or visual alert signal to its affiliates. Moreover, from personal observations by the sampler, all the individuals which did not respond to the procedure were sampled above the water surface at a medium-high speed (approximately 12knots), which might have concealed, given all the water splash and social interaction happening at the same time, that the biopsy sample was taken from their affiliate, and thus influence the groups' reaction, or lack of it. Therefore, it is possible that most group reactions recorded were a response to the targeted animal's movements as opposed to a reaction to the bolt strike. As it has been found in other mammals, this study supports the idea that the social context seems to influence the stress response behaviour (Kappeler, Barrett, Blumstein & Clutton-Brock, 2013).

There were no strenuous reactions recorded from any of the individuals targeted (as in Hooker et al., 2001). The few strong responses that have been reported in previous studies were generally linked to the dart not detaching immediately off the animal's body, to entanglement of retrieval lines or due to a strong impact (Bearzi, 2000; Krützen et al., 2002). It may be possible that, since this technique does not allow for the biopsy tip to stay attached to the animal's body and only allows for a brief duration of contact, it prevents strong reactions. Also, given that this method requires close approximation, extra force is not required to acquire the sample as in remote methods, hence the impact being much lower. Additionally, it is important to highlight that this method requires the animals to closely bow-ride, which in itself can be highly demanding since the dolphin has to continuously focus on both the boat's directional movements and speed as well as its affiliates activities and itself. By coordinating it all, adding to the vessel's obvious disturbance, the target animal might have simply too many distractions already happening to it to properly perceive the biopsy sampling, thus preventing stronger reactions from being displayed. However, respecting the Peale's dolphin there was a record of one strong response by a non-targeted individual (the targeted animal associated with this affiliate reacted simply in a mildly way). That one vigorous reaction happened in the disturbed area of Castro. It is interesting to remark how though most group reactions were of light intensity, we could still find animals that would react strongly. The reason for its expressive response was not clear but it only lasted for 1 minute, while the animal was swimming away from the boat. Overall, and although the lack of strong responses can lead us to assume this minimally invasive method is safe for the animals and the researchers on board, there is always the risk for serious impacts on the animals' welfare, thus emphasizing the importance of critical careful planning and review of any research method.

There was a tendency for larger assemblies of animals to react less intensively to the procedure on both species. It also seemed more likely to obtain reactions of lighter intensity in groups which were travelling. This could be due to the fact that the research boat would pair up with the dolphins' travelling speed, which was kept constant throughout the whole procedure. The continuity of this behavioural state seemed to predispose the animals to bow-ride. Which in turn, besides facilitating biopsy sampling, may have also resulted in a lower stress perception by the dolphins leading to a lighter intensity response to it.

In general, the response magnitude appeared to decrease from an individual level to a group level, particularly when animals were found socializing before the procedure, however most biopsy samplings were collected while the animals were milling, which resulted in mild behavioural responses. It was also apparent that mild reactions (level 2) occurred when the water visibility was poor (<4.5 m). Even though a variance of only a few meters appeared to display such a difference between the proportion of mild reactions compared to startle or no responses, and that possibly the vision isn't the most used sense on these species whose habitat is highly productive in organic matter (Heinrich, 2006) there might be some explanation to these distinct behaviours amongst dolphin groups. For instance, in an environment with high visibility, a group of animals (not targeted) that receives an acoustic alarm signal from a conspecific, can visually confirm the absence of danger and decide its behavioural response based on either of the cues, visual or acoustic. Whereas in an environment with low visibility range, the dolphins might not be able to visually perceive its surroundings, relying solely on acoustic signals and thus possibly reacting exclusively based on the targeted dolphin's acoustic distress warning.

We noted how all individual Peale's dolphin who did not respond to the procedure were monitored prior to sampling for less than 35min and the groups which also didn't react were monitored for a period under 25min, suggesting that maybe the best time-frame for pre-monitoring would be under 25 minutes. Furthermore, we observed how the longest encounter durations (>1h) had a bigger proportion in mild reactions (level 2) on both individual and group levels on the Peale's dolphin, implying the amount of time spent with the groups may elicit stronger reactions. Lastly, we also noted that all individual Chilean dolphins reacted strongly (level 2) at Beaufort 0, which could be because with a mirror-like sea it becomes easier to perceive the visual cues.

In our study, we found no variable that would expressively determine the dolphin's response to the procedure, as it is a multifactorial process as well as dependent upon intraspecies individual-variation. Our sample size was also quite limited to allow inferences. Nonetheless, we did slightly raise the veil on some factors that can influence the success of the technique (Appendix 6) and its implication on the species welfare, which we will try to incorporate in future studies.

4.2.2 - Behavioural considerations

Animals who present restricted plasticity, are likely to be more vulnerable in a constant changing environment, enhanced now by human interference. The Chilean dolphin besides only being confined to the waters of Chile, also presents high site fidelity, making it especially vulnerable to the effects of human activities and habitat interference and degradation (Viddi et al., 2015). As discussed in the literature review and presented in the current study, the Chiloé marine ecoregion has become under increasing pressure as a result of intensive fishing activities, such as salmon and mussel farming, coastal development and marine transportation traffic. Frequently a change in behaviour is the primary response to human-altered conditions which allows the species to adjust to the new environmental conditions, thus increasing its fitness (Wong & Candolin, 2015). Still it is vital the animals present some degree of behavioural plasticity to increase its resilience.

It is interesting to note that overall the Chilean dolphin always reacted to the technique and showed to be quite consistent in the degree it responded, both at an individual and group levels. This species presents fairly limited movements, low levels of long-term association between individuals, and usually occur in small groups (Prox, 2017). The coincidental results between the Chilean dolphin's individual and group reactions might be a combination of all these factors added to their particular elusive behavior (less surface active), all of which contribute to an overall limited behavioural plasticity. Admitting how important behavioural flexibility can be for a species survival, it becomes clear that more behavioural and ecological studies on this species are imperative in order to understand how to promote the conservation of the Chilean biodiversity. The *Lagenorhynchus* genus, on the other hand, is known to have some variation in social structure, has been found engaged in cooperative feeding in mixed groups and has a wider habitat range (Heinrich et al., 2010). This might explain the diversity of reactions recorded to the biopsy sampling and also the larger group behavioural repertoire registered. It is explicit that in general there was a noteworthy variance between species, but unfortunately the current knowledge on both genera is still deficient, thus underlining the relevant contribution studies like this can bring to future research methodologies.

In sum, the results obtained suggest that if proper care and caution are used, biopsy sampling of the Chilean and Peale's dolphins is not likely to produce intense short-term behavioural changes. The method is economical, easy to employ, and fast to repeatedly deploy it. Additionally, in situations where remote biopsy riffles or crossbows aren't practical or available this could be an efficient alternative.

5 - Conclusions

In contemporary research, biopsy sampling has become one of the most relevant tools in the study of cetaceans' physiology, health, biology and ecology. It is especially important when involving a threatened or endangered population, where obtaining accurate information is vital for the development of conservation strategies (Weller et al. 1997; Gorgone et al., 2008).

Even though it seems a paradox, when researching these species we aim to protect, we contribute to some extent to the impairment of their welfare. This is because in order to investigate the conservation issues affecting the species and change policies, we many times forgo the individual welfare (de Vere et al., 2018).

In wildlife research, quality data is a vital requirement to obtain valuable results and reach proper conclusions from where to develop good management decisions. This intrinsically depends upon the quality of the animals under study which means the welfare of the free-ranging ones has to be promoted (Castle et al., 2016).

When sampling free-ranging marine mammals, the least invasive method should be preferred (Cunha et al. 2010). It is only possible to sample with a pole species that allow for a close approach and/or bow-ride. The pole method was selected due to these species size (< 2 meters in length) and its elusive behaviour, which limits their surface period and consequently our sampling technique time window. Although the pole seems to achieve low impact sampling, it may cause more stress due to the need for a closer approach and longer chasing time.

Overall it is an easy and economical method, fast to set up on each trial, thus allowing sampling to follow on a short timed sequence and it does not require the targeted animal to surface, since it can be biopsied while still underwater (< 50 cm deep). This increases the chances for the sampler to biopsy the animals and it may be a very useful method in situations where remote biopsy systems aren't feasible (Bilgmann et al., 2007; Loizaga de Castro et al., 2013).

The results obtained suggest that, if proper care and caution are used, biopsy sampling of the Chilean and Peale's dolphins is not likely to produce intense short-term behavioural changes. Our biopsy technique produced mainly mild short term reactions, which is consistent with previous studies using the same method (Marsili & Focardi, 1996; Fossi et al., 2004; Bilgmann et al., 2007; Loizaga de Castro et al., 2013) and using remote ones (crossbows: Weller et al., 1997; Hooker et al., 2001; Gorgone et al., 2008; Jefferson & Hung, 2008; Cunha et al. 2010; Fruet et al., 2017; and riffles/pneumatic guns: Barrett-Lennard et al. 1996; Krützen et al., 2002; Parsons et al. 2003; Tezanos-Pinto & Baker, 2012).

If this disruption is caused by the momentarily painful contact or as a result of a surprising stimulus, we could not assess. And even though biopsy sampling also elicits unintended disturbance on behaviour of nearby non targeted animals, overall effects appeared to be minimal.

Nonetheless, while the behavioural reactions of cetaceans studied to date have been mild to moderate and of limited duration, the technique is still biologically invasive. Therefore, efforts such as those of Brown et al. (1994), Patenaude & White (1995), and Gauthier & Sears (1999) to refine sampling procedures, reduce physiological trauma to the target animal and increase sample collection effectiveness are critically important.

Our observations support that this procedure can be used as a safe tool, resulting in a low short-term impact on these species, although we defend a long-term monitoring is required to better understand the impact of such technique on the population's dynamics.

6 - Limitations to this study

This was a preliminary study and it is our intention to gather data from a larger sample in future work to achieve a greater statistical power.

In fact, the biggest limitation of our study was the sample size. However, the number of surveys was limited by several factors: the weather conditions (that particular area is quite windy and unpredictable, and we could only go out when Beaufort < 4), financial resources, logistical difficulties and mechanical problems. Besides, the high levels of water turbidity and speed currents make it difficult to predict the surfacing behaviour of dolphins (Fruet et al., 2017).

We only included in the analysis biopsy attempts in which we had recorded videos to confirm our field observations. To properly assess the animal's behavioural response, the video discernibility had to be good, but given the species elusive behaviour, their lack of natural marks to allow correct identification, the poor visibility of the water and our elementary video material, we frequently lacked good quality documentation of the events.

Data can be affected by variation in sample collection, handling, and processing, which can be challenging to regulate under field conditions. Difficulty in controlling the hit location and penetration depth of the biopsy tip, may too have affected the results.

Additionally, it may be important to consider individual variation in behaviour towards the boat, since sampling with a pole requires a close approach of the animals and hence invalidate the equal probability of sampling. This is why Cunha et al. (2010) refer the need of using a projector (crossbow, air gun or riffle), seeing it is a remote method. Nevertheless, the sound transmission underwater is excellent, and approaching boats can be audible from a far distance (Nowacek, Thorne, Johnston & Tyack, 2007). This may also suggest that it is possible to miss dolphin groups' reactions if they avoid the boat even before we get to detect their presence (Peters et al., 2013). The boat's speed, manoeuvring, and angle of approach are all significant factors to consider, seeing that the animal's disruption increases with high impact approaches (Constantine, Brunton & Dennis, 2004; Lusseau, 2006). Animals seem to be specially affected by high speed vessels (Sai Leung & Leun, 2003) not only due to its fast approach but also due to the higher frequency noise its motors and auxiliary machinery produce.

According to Mann (1999), the follow protocol and behavioural sampling method chosen can also have a substantial effect on the amount and quality of the animal behaviour we are able to record and later analyse. It may be possible that scientists are missing key animal reactions to human disturbance, and hence the importance of characterizing and standardizing such techniques. We monitored the groups for longer than 30min which is considered a group-follow protocol. Following a group may result in biases, given the observer's attention will naturally be drawn to either more expressive animals or more expressive behaviours, such as breachings instead of directional changes for example. A rigorous systematic sampling can be difficult, therefore researchers need to admit rules for common situations (Mann & Würsig, 2014). This would be, for example, in the case the dolphin group splits during the observation, the team should stay with the bigger group and continue the behavioural recording.
We noted the group behaviour prior to biopsying using a Focal Group Sampling, where we'd consider the behaviour being performed by more than 50% of the group. It is important we recognize the observer could've not continuously observe all the animals equally, plus this would mean they would need to be visible at all times, which is obviously impossible. This, however, is a limitation on marine mammal ethology.

7 - Future Recommendations

We couldn't gather enough statistical power with the number of animals sampled, so it would only be beneficial to extend our sample size.
Gorgone et al. (2008) pointed out how the animals' reaction depends on various factors, such as sea state, season, number of vessels present and distance between dolphins in a group (tightness). Peters et al. (2013) referred that the disturbance of the vessel speed, the manoeuvring and angle of approach might influence the dolphins' responses to the procedure. Crossing the group's path, approaching it closely and at high speed, will generally increase the animals' disruption, in a so called "high impact approach".
Vessel traffic and tourism boats are usually linked with marine mammals spending less time resting and feeding which leads to poor physical fitness and chronic stress (de Vere et al., 2018). Analysing if the way we approach these dolphins for biopsy sampling can influence their reaction, and if there is a cut-off, from when we should stop chasing them in order to avoid a moderate/strong reaction would be interesting. By being able to identify the circumstances in which the dolphins tend to react more severely, we can try to further reduce them, by acting preventively.
In the future it would also be interesting to assess long term impacts of the biopsy sampling in the populations targeted. This requires regular surveys to determine re-sighting rates of the targeted dolphins and, if possible, non-targeted dolphins as well. Likewise, the evaluation of

their behavioural states, before and after sampling, would be necessary to assess the changes we provoke on their budget time for the different activities. All these studies will help to better understand the cumulative effects of this sampling method in the group of dolphins, and not simply the individual sampled.

Analysing wound healing in these species could be really important, in order to corroborate the findings on other cetaceans, where the wounds show already superficial cover of the hit point after the first month post-biopsy, with no infection evidences. This would support the theory that biopsy sampling is not detrimental to the dolphin’s health status.

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Appendix 1 – Beaufort Wind Scale.

Accessed on March 3th, 2017 from: http://www.spc.noaa.gov/faq/tornado/beaufort.html

Force	Wind (Knots)	WMO Classification	Appearance of Wind Effects on the water
0	Less than 1	Calm	Sea surface smooth and mirror-like
1	1-3	Light Air	Scaly ripples, no foam crests
2	4-6	Light Breeze	Small wavelets, crests glassy, no breaking
3	7-10	Gentle Breeze	Large wavelets, crests begin to break, scattered whitecaps
4	11-16	Moderate Breeze	Small waves 0.3-1.2 m becoming longer, numerous whitecaps
5	17-21	Fresh Breeze	Moderate waves 1.2-2.4 m taking longer form, many whitecaps, some spray
6	22-27	Strong Breeze	Larger waves 2.4-4 m, whitecaps common, more spray
7	28-33	Near Gale	Sea heaps up, waves 4-5.8 m, white foam streaks off breakers
8	34-40	Gale	Moderately high (5.8-7.6 m) waves of greater length, edges of crests begin to break into spindrift, foam blown in streaks
9	41-47	Strong Gale	High waves (7-9.8 m), sea begins to roll, dense streaks of foam, spray may reduce visibility
10	48-55	Storm	Very high waves (8.8-12.5m) with overhanging crests, sea white with densely blown foam, heavy rolling, lowered visibility
11	56-63	Violent Storm	Exceptionally high (11.3-15.8 m) waves, foam patches cover sea, visibility more reduced
12	64+	Hurricane	Air filled with foam, waves over 13.7m, sea completely white with driving spray, visibility greatly reduced.

Apendix 2 – Table with p values obtained for each predictor categorical variable using Fisher's exact test. Significance at a p value < 0.05.

FISHER'S EXACT TEST	CATEGORICAL VARIABLES	P VALUE
INDIVIDUAL REACTION	Area	0.096
	Behaviour	0.592
	Dispersion	0.462
	Geography	0.171
	Group size	0.070
	Season	0.300
	Species	0.018 *
	State	0.943
GROUP REACTION	Area	0.246
	Behaviour	0.619
	Dispersion	0.825
	Geography	0.104
	Group size	0.234
	Season	0.550
	Species	0.0006 *
	State	0.773

Appendix 3 – Frequency table from all the categorical predictive variables for the Chilean dolphin.

Variable	Levels of variable	Absolute frequency	Relative Frequency	Relative Cumulative Frequency
Area	Calbuco	8	25,0	25,0
	Castro	4	12,5	37,5
	Melimoyu	11	34,4	71,9
	Quellon	5	15,6	87,5
	Quemchi	1	3,1	90,6
	Renihue	3	9,4	100,0
	Total	32	100,0	-
State	Natural	14	43,8	43.8
	Disturbed	18	56,2	100,0
	Total	32	100,0	-
Geography	Continental	22	68,8	68,8
	Insular	10	31,3	100,0
	Total	32	100,0	
Year	2015	10	31,3	31,3
	2016	13	40,6	71,9
	2017	9	28,1	100,0
	Total	32	100,0	-
Season	Fall	19	59.4	59.4
	Summer	13	40.6	100,0
	Total	32	100,0	-
Size	Small	6	19.4	19.4
	Medium	17	54.8	74.2
	Large	8	25.8	100,0
	Total	31	100,0	-
Dispersion	Mixed	17	73,9	73,9
	Tight	6	26,1	100,0
	Total	23	100,0	-
Behaviour	Milling	11	47,8	47,8
	Socializing	6	26,1	73,9
	Travelling	6	26,1	100,0
	Total	23	100,0	-
Human Structures	Aquaculture	2	11.1	11.1
	Clam boat	4	22.2	33.3
	Fishing boat	2	11.1	44.4
	Mussel Farm	4	22.2	66.6
	Salmon Farm	6	33.3	100,0
	Total	18	100,0	-

Appendix 4 – Frequency table from all the categorical predictive variables for the Peale's dolphin.

Variable	Levels of variable	Absolute frequency	Relative Frequency	Relative Cumulative Frequency
Area	Calbuco	9	12.0	12.0
	Castro	11	14.7	26.7
	Melimoyu	19	25.3	52.0
	Quellon	16	21.3	73.3
	Quemchi	5	6.7	80.0
	Renihue	15	20.0	100.0
	Total	75	100.0	-
State	Natural	34	45.3	45.3
	Disturbed	41	54.7	100.0
	Total	75	100.0	-
Geography	Continental	43	57.3	57.3
	Insular	32	42.7	100.0
	Total	75	100.0	-
Year	2015	23	30.7	30.7
	2016	38	50.7	81.3
	2017	14	18.7	100.0
	Total	75	100.0	-
Season	Fall	54	72.0	72.0
	Summer	21	28.0	100.0
	Total	75	100.0	-
Size	Small	19	26.4	26.4
	Medium	32	44.4	70.8
	Large	21	29.2	100.0
	Total	72	100.0	-
Dispersion	Mixed	43	65.2	65.2
	Tight	23	34.8	100.0
	Total	66	100.0	-
Behaviour	Chasing	11	16.4	16.4
	Milling	30	44.8	61.2
	Socializing	14	20.9	82.1
	Travelling	12	17.9	100.0
	Total	67	100.0	-
Human Structures	Aquaculture boat	16	44.4	44.4
	Clam boat	2	5.6	50.0
	Fishing boat	2	5.6	55.6
	Mussel Farm	11	30.6	86.2
	Salmon Farm	5	13.8	100.0
	Total	36	100.0	-

Appendix 5 - Absolute frequencies of dolphin groups (Ce & La) sighted per area studied. "Human" represents the number of groups found near human structures. The mean distance expresses, in meters, the average distance such groups were found from human activities.

			Natural		Disturbed			
Species	Year		Melimoyu	Reñihue	Calbuco	Castro	Quellón	Quemchi
Chilean dolphin	2015	N total	2	-	-	9	99	-
		N human	2	-	-	6	79	-
		$\bar{X}$ distance	441	-	-	311.2	203.2	-
	2016	N total	15	5	-	1	47	-
		N human	4	2	-	1	31	-
		$\bar{X}$ distance	411	506	-	187	261	-
	2017	N total	-	-	41	-	-	12
		N human	-	-	23	-	-	4
		$\bar{X}$ distance	-	-	260.2	-	-	426.3
Peale's dolphin	2015	N total	17	-	-	14	79	-
		N human	8	-	-	9	46	-
		$\bar{X}$ distance	614.5	-	-	259.3	486.8	-
	2016	N total	18	10	-	8	31	-
		N human	1	5	-	6	22	-
		$\bar{X}$ distance	728	227	-	365	441	-
	2017	N total	-	-	29	-	-	14
		N human	-	-	14	-	-	4
		$\bar{X}$ distance	-	-	277.7	-	-	385.5

Appendix 6 - Variables that appeared to positively influence the dolphin's reaction and the overall success of the procedure.

Positive Influence Variables	**Chilean dolphin**	**Peale's dolphin**
Water depth	< 10 m	no influence
Duration of encounter	no influence	< 1h
Group size	large ≥ 10 individuals	large ≥ 10 individuals
Pre-biopsy predominant behaviour	socializing	travelling
Pre-biopsy monitoring period	no influence	< 30min
Visibility of the water	< 4m	> 5m

Appendix 7 – Scientific communications list

- Fragoso, Mariana; Espinosa-Miranda, Cayetano; Fuentes-Riquelme, Marjorie (2017) Short-term behavioural reactions of Peale's dolphins (*Lagenorhynchus australis*) and Chilean dolphins (*Cephalorhyncus eutropia*) to a biopsy pole system in the Chiloense Marine Ecoregion, southern Chile. VI FAUNA International Conference. Lisbon University, 11th November 2017. Oral communication.

- Fragoso, Mariana.; Espinosa-Miranda, Cayetano.; Fuentes-Riquelme, Marjorie. (2016) - Short-term behavioural reactions of two dolphin species to a biopsy pole system in Chiloense Marine Ecoregion, southern Chile. 3rd EAZWV Iberian Section Meeting. Lisbon Oceanarium, 11th and 12th November 2016. Panel communication